Antioxidant adaptations in liver fibrogenesis

Tiebosch, Marjolein

Antioxidant adaptations in liver fibrogenesis

M.H. Tiebosch

The research described in this thesis was conducted at the Department of Hepatology and Gastroenterology, Centre for Liver, Digestive, and Metabolic diseases, University Medical Centre Groningen, University of Groningen, Groningen, the Netherlands.

Research described in this thesis was funded by grants from:
Junior Scientific Masterclass (JSM), University Medical Centre Groningen
J.C. de Cock stichting

Printing of this thesis was financially supported by:
Rijksuniversiteit Groningen
De Nederlandse Vereniging voor de Hepatologie
Graduate School of Medical Sciences (Groningen University Institute of Drug Exploration)

Their contribution is gratefully acknowledged.

Printed by: Gildeprint, Enschede, The Netherlands
Layout: Nicole Nijhuis, from Gildeprint, Enschede, The Netherlands

ISBN printed version: 978-94-6108-635-8
ISBN digital version: 978-94-6108-639-6

rijksuniversiteit
groningen

Antioxidant adaptations in liver fibrogenesis

Proefschrift

ter verkrijging van de graad van doctor aan de
Rijksuniversiteit Groningen
op gezag van de
rector magnificus prof. dr. E. Sterken
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

woensdag 16 april 2014 om 16.15 uur

door

Marjolein Henriëtte Tiebosch

geboren op 29 juli 1987
te Maastricht

Promotores
Prof. dr. A.J. Moshage
Prof. dr. K.N. Faber

Beoordelingscommissie
Prof. dr. M. Arrese
Prof. dr. R.H. Henning
Prof. dr. H. Van Goor

Aan mijn ouders

Paranimfen

Manon Buist-Homan

Esther M. Verhaag

Table of Contents

Chapter 1

General Introduction

Liver fibrosis and oxidative stress

MH Tiebosch, KN Faber, H Moshage

Liver disease prevalence and etiology

Liver fibrosis represents a large health burden for modern society. In 2012, 806 deaths were reported as a consequence of liver disease in general in the Netherlands.(1) Furthermore, since liver fibrosis is a chronic condition, that may progress to cirrhosis, it also has a high morbidity, which leads to hospital admissions. This morbidity is mostly due to complications (i.e. portal hypertension leading to esophageal varices, hepatorenal syndrome) and these also markedly decrease both the patients' quality of life as well as life expectancy.(2) Currently, there is no effective therapy for liver fibrosis and cirrhosis except for liver transplantation.

Liver fibrosis and eventually cirrhosis is most commonly caused by chronic liver diseases such as alcoholic liver disease and viral hepatitis B and C, but also fatty liver disease (non-alcoholic fatty liver disease or NAFLD and non-alcoholic steatohepatitis or NASH), primary sclerosing cholangitis, primary biliary cirrhosis etc. Nowadays fatty liver disease emerges as an common etiological factor for liver fibrosis, as the incidence of obesity increases. Recently, it was shown that 27% of overweight people (BMI>25) had NAFLD compared to 7% in the lean (BMI<25) group.(3)

Acute liver disease, for example due to acetaminophen intoxication or ischemic hepatitis, rapidly leads to liver failure, but when there is no chronic harmful stimulus liver fibrosis and cirrhosis do not develop.

Liver anatomy

The liver is composed of several liver lobes which are further subdivided into lobules, the functional subunits of the liver. The liver has a dual blood supply: on the one hand oxygen-rich blood from the hepatic artery, and on the other hand nutrient-rich blood from the portal vein, which originates in the mesentery of the gastrointestinal tract. The portal vein is most important in the blood supply to the liver, accounting for approximately 75% of the blood flow.

The liver lobule

A lobule is a hexagonal structure. On every corner a branch of the hepatic artery and a branch of the portal vein enter, and in the middle of the lobule there is a central draining vein **(Figure 1)**.

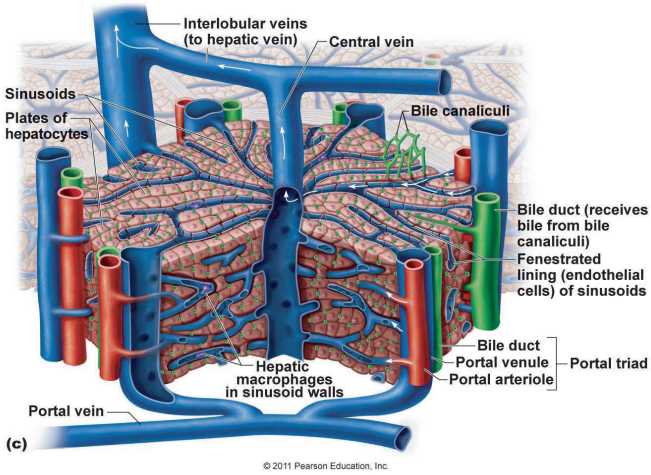


Figure 1. A schematic overview of a liver lobule.
A schematic overview of the portal triad consisting of the portal vein (blue), hepatic artery (red) and draining bile duct (green) with the draining central vein in the middle of the hexagonal structure of the liver lobule. Taken from cms.webstudy.com.

Both the arterial and the venous portal blood run through capillaries, called sinusoids, towards the central vein. **(Figure 2)** These sinusoids are comprised of fenestrated endothelial cells, allowing nutrients to leave the blood flow, passing through the space of Disse (the space between sinusoidal epithelium and the hepatocytes), where they then can be absorbed at the basolateral membrane of the hepatocytes. Metabolized nutrients (amino acids, glucose etc) or proteins produced by the hepatocyte also can be excreted to the blood from the basolateral side. On the other side of the hepatocyte, at the canalicular membrane, various products can be excreted by the hepatocytes, such as (waste) metabolites, or newly synthesized proteins or hormones and bile acids. All these excreted products end up in the bile canaliculus. The bile canaliculi merge into the common hepatic bile duct, which connects to the intestine after the ductus cysticus (connecting to the gallbladder) joins to form the common bile duct.

Apart from sinusoidal endothelial cells and hepatocytes, other cells present in the sinusoids include the Kupffer cells and hepatic stellate cells. Kupffer cells are the residential macrophages of the liver.

Hepatic stellate cells reside in the space of Disse. Portal myofibroblasts are believed to be located in the portal areas, along the portal vein. These two cell types will be discussed in more detail in later paragraphs.

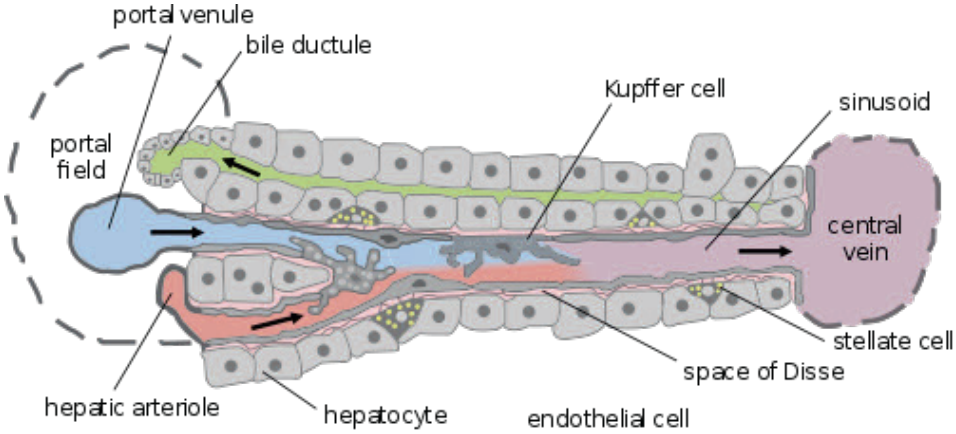


Figure 2. A schematic representation of a liver sinusoid.
Both arterial and portal venous blood flows from the portal field to the central draining vein through capillaries, called sinusoids. The fenestrated epithelium along these sinusoids allows exchange of oxygen, nutrients and waste products between the blood and the hepatocytes. The bile ducts are formed by the canalicular membranes of the hepatocytes, draining bile back to the portal field, transporting bile acids produced by the hepatocytes via the gallbladder to the intestine. Other cells present in the sinusoid are the liver-specific macrophages, Kupffer cells, and hepatic stellate cells. Taken from www.mananatomy.com

Fibrogenesis

Upon the infliction of injury (wounding), a wound-healing response starts in order to contain and recover the damage. This occurs in liver disease as well, irrespective of the nature of the damaging stimulus. After acute liver injury, a beneficial wound-healing mechanism regenerates damaged hepatocytes, replacing necrotic and apoptotic cells.

However, when the cause of injury persists, excessive scar tissue (extracellular matrix) is deposited and fibrosis develops. The cell types responsible for the matrix production and deposition are the myofibroblasts. Myofibroblasts can originate from different sources, of which the hepatic stellate cell is the best known. In the next paragraph these and other cell types contributing to extracellular matrix production will be discussed.

Hepatic stellate cells

Hepatic stellate cells (HSCs) are the main cell type involved in extracellular matrix production. In a non-diseased quiescent state, they are responsible for vitamin A storage in phenotypically characteristic fat droplets. HSCs represent 5-8% of total liver cells, and they can store up to 70% of total retinoid content in the healthy adult in the form of retinyl esters.(4)

Upon liver damage, irrespective of the etiology, hepatic stellate cells will become activated and start to proliferate. This activation process consists of fast and drastic morphological and functional changes.(5) Several factors can stimulate HSC activation: profibrogenic factors of which TGF-β1 is believed to be the most potent, but also cytokines released from immune cells and damaged or dying hepatocytes.

Activation of HSCs can be divided into an initiation and a perpetuation phase.(6) Initiation comprises all the events leading to a phenotypically changed hepatic stellate cell, the myofibroblast. Losing fat droplets is the key feature of this initiation phase. Whether this is a cause or a consequence of HSC activation is unknown. However, it is observed that when the adipogenic phenotype is maintained, there is less fibrosis.(7,8) In this initial phase, HSCs change their metabolism from oxidative phosphorylation to glycolysis, although retaining large numbers of mitochondria. This is known as the Warburg effect, which is also commonly seen in cancer cells. It was shown that the accumulation of lactate was vital for transdifferentiation of quiescent HSCs to activated HSCs.(9)

In the perpetuation phase, the activated stellate cell is primed for the different tasks that it now has to fulfill as a myofibroblast: the development of scar tissue and containment of injured tissue. In this phase the hepatic stellate cells start to proliferate upon both autocrine (via platelet derived growth factor or PDGF) and paracrine stimuli. The cells are able to migrate to a site of injury via chemotaxis, and they produce extracellular matrix, most prominently collagen type I. As hepatic stellate cells are myofibroblasts after their conversion, they are also able to contract. This causes vasoconstriction, which contributes to portal hypertension.

Stellate cells also play a large role in the inflammatory response, both as a modulator and as an effector. Like inflammatory cells, activated stellate cells express chemotactic cytokines and engage in inflammatory signaling. For example via toll-like receptor 4 (TRL4) HSCs can be stimulated to produce monocyte-attracting protein 1 (MCP1 or CCl2) thereby contributing to a pro-inflammatory microenvironment.(10) Besides this modulating role, there are reports claiming that hepatic stellate cells have the capability to phagocytose apoptotic bodies. This induces a profibrogenic response as well as a survival signal in the HSCs.(7,11-13)

Portal myofibroblasts

Another source of extracellular matrix proteins, and therefore significantly involved in fibrogenesis, are the portal myofibroblasts (PMFs).(14) However, their contribution and even their existence as a different cell type besides the HSC is heavily debated. As they are located around the portal tract, they are believed to participate in cholestatic liver disease, contributing to bile duct proliferation.(15) Several groups have compared HSCs and PMFs, in order to identify PMFs as a distinct cell type. Tuchweber *et al.* found differences in the expression of fibroblast markers alpha-smooth muscle actin (αSMA) and desmin when comparing PMFs and HSCs; PMFs were desmin-negative and αSMA-positive, whereas HSCs were desmin- and αSMA-positive. This was confirmed in another study, where desmin and/or αSMA positivity and negativity were studied in two models of liver disease, arterial ischemia and cholestasis by BDL.(16) A proteomics study from 2010 observed that cytoglobin, a peroxidase important in eliminating reactive oxygen species, was predominantly expressed in HSCs and not in PMFs. A marker for PMFs was phosphorylated cofilin (p-cofilin, a regulator of stress fiber formation), which was absent in HSCs. This observation led to the hypothesis that PMFs are more involved in the production of extracellular matrix, whereas HSCs are more involved in protecting hepatocytes.(17) A specific marker for PMFs is not yet available, therefore it is difficult to show the relative contribution and importance of these distinct cell types in liver fibrogenesis. Culturing both PMFs and HSCs, we found a striking difference in their ability to proliferate. Hepatic stellate cells proliferate for about 14 days after isolation, whereas portal myofibroblasts are able to proliferate up until passage 8 (unpublished data).

As described above, HSCs and PMFs are the main contributors to extracellular matrix (ECM) production in fibrosis.(18,19) However, other cells may also play a role in this fibrosis-defining process, such as bone marrow-derived cells. However, these cells comprise a small population, which is only observed in experimentally-induced liver disease and their contribution to ECM production is unknown.(20)

Epithelial-mesenchymal transition

In literature, the involvement of epithelial-mesenchymal cell transition (EMT) in fibrosis is proposed. This implicates that cells from epithelial origin can transdifferentiate, like HSCs, into (myo)fibroblasts that belong to the mesenchymal cells. In the lung and kidney there is evidence for a contribution of EMT to fibrosis.(21,22). However, this is only a theoretical concept in liver fibrosis, since it was not yet shown in liver disease.

Extracellular matrix

In fibrosis, the extracellular matrix (ECM) is not only altered quantitatively, but also qualitatively.(23) There is an increase in the amount of fibrillar collagen type I and III, proteoglycans, fibronectin and hyaluronic acid.(24-26) Deposition of this ECM in the liver, especially in the sinusoidal area, will lead to a distortion of normal architecture. As a consequence, there is impairment of the nutrient and metabolite exchange between the sinusoidal area and the hepatocytes. Moreover, blood flow is impaired, which explains the commonly seen complication of portal hypertension in liver cirrhosis patients.

While the production of extracellular matrix is increased, the degradation of ECM is also inhibited by the expression of tissue inhibitor metalloproteinases (TIMPs). These yield a powerful profibrotic effect over counteracting metalloproteinases (MMPs), which normally degrade newly formed ECM.(27) TIMPs thereby protect newly-produced matrix even though MMPs are present in high concentrations.(28,29) Thus, the TIMP/MMP balance is important for the progression of disease.

Reversal of fibrosis

When the balance is shifted in favor of MMPs, reversal of fibrosis can occur. Spontaneous resolution of fibrosis in patients has been reported.(30) In animal models advanced fibrosis can also be resolved spontaneously, within 4-6 weeks.(31) During this period the liver tissue undergoes architectural remodeling, in which the fibrotic matrix is degraded. Moreover, there is spontaneous apoptosis of myofibroblasts as they lose their survival signals in a MMP-rich microenvironment.(32,33) There might be an important role for macrophages in this process as well, since they can produce MMPs.(34,35)

End stage liver cirrhosis is an irreversible disease. Extensive remodeling of the extracellular matrix is possible if the damaging stimulus is halted, but the large mature septa (consisting of collagen type I and elastin) fail to recover. These elastin-rich areas appear to have crosslinks mediated by transglutaminase, which are highly resistant to enzymatic degradation. Additionally, these mature scars contain few cells, which probably also results in less susceptibility to degradation.(36)

Inflammatory cells and fibrogenesis

As fibrosis is the result of an exaggerated wound healing response, inflammation plays a large role. Upon the initiation of injury, several immune cells are recruited to the site

of injury: neutrophils, macrophages (both from resident and bone marrow-derived populations) and lymphocytes.

Neutrophils, as part of the innate immune system, are one of the first cells to arrive at the site of injury. A variety of molecules released from damaged cells, collectively called damage-associated molecular patterns (DAMPs), attract neutrophils. Examples of DAMPs are: deoxyribonucleic acid (DNA), both nuclear and mitochondrial, adenosine triphosphate (ATP), cytochrome c, heat shock protein, and hedgehog ligands.(37) Other molecules, both organic and inorganic can also cause sterile inflammation such as asbestos, urate crystals, cholesterol and amyloid B.(38) Upon sensing these DAMPs, neutrophils adhere in the sinusoids and migrate through the sinusoids to the site of injury on a chemokine gradient.(39)

This first innate reaction is non-specific, and therefore it can quickly lead to a widespread inflammation with activation of other immune cells, even if the original tissue injury was limited.

Previously, it was shown that upon partial depletion of neutrophils, fibrosis still developed in an *in vivo* cholestatic liver disease model.(40) This indicates that other inflammatory cells are more important in fibrogenesis, possibly the macrophages.

Macrophages play a crucial role in inflammation.(41) They can be subdivided into two populations, the resident and the infiltrating macrophages. Resident macrophages in the liver are called Kupffer cells and reside in the sinusoids. They are believed to patrol the sinusoids in order to detect any injuring stimulus or invading microorganism in an early phase. The Kupffer cells have been shown to recognize DAMPs and, in response, produce the proinflammatory interleukin 1-beta (IL-1β) and other cytokines, thereby attracting other cells to the site of injury, such as neutrophils.(42) In addition, Kupffer cells can directly phagocytose the damaging stimulus. Monocytes derived from the blood can invade the liver when sensing cytokines and chemokines secreted by either neutrophils or Kupffer cells, and then differentiate into macrophages.(43,44)

Classically, macrophages are known for their phagocytosing capacities, which mean that they engulf and digest cell debris, pathogens or other potentially harmful materials. Phagocytosis can be both pro- and antifibrotic, for example phagocytosis of dead cells can lead to production of profibrotic cytokines, such as TGF-β1 and platelet-derived growth factor (PDGF), but it also removes profibrotic stimuli produced by the dead/dying cell itself. In addition, the inflammatory response from macrophages can be both pro- and antifibrotic: the recruitment of inflammatory cells stimulates fibroblasts, but

inflammation is terminated by cleaning up cellular debris. Moreover, deposition of extracellular matrix is controlled by macrophages through the removal of apoptotic myofibroblasts, and the direct elimination of excess collagen deposition.

Since macrophages can play a dual role in the wound healing response, it has been hypothesized that there are different phenotypes of macrophages involved in fibrogenesis and the resolution of fibrosis. On the one hand there is the classic, pro-inflammatory phenotype also called the M1 phenotype, known for its phagocytosing capabilities. On the other hand there is the M2 phenotype, induced upon alternative activation, which is characterized by a more profibrogenic phenotype.

Therefore, it can be concluded that macrophages play a central role in fibrogenesis. This is further confirmed by experiments where macrophages were selectively depleted from the liver; Duffield *et al.* showed reduced fibrosis in ongoing liver injury.(45) Also in animals deficient in the principal macrophage chemokine CCL2-CCR2-axis, accompanied with a reduction of monocyte/macrophage infiltration upon chronic hepatic injury, a protection against fibrogenesis was shown.(46)

T lymphocytes are also involved in inflammation and can orchestrate the adaptive immune response via the different T-helper (Th) cell phenotypes. However, they can also influence the macrophage phenotype, as they can produce different sets of cytokines. For example, a switch from Th2 to Th1 cytokines in CCl_4-induced liver injury conferred protection against fibrosis.(47) Also Th2 cytokines can directly stimulate fibroblasts to synthesize collagen.

Reactive oxygen species

Oxidative stress plays an important role in fibrogenesis and the accompanying inflammation. Oxidative stress is defined as the inappropriate exposure to reactive oxygen species (ROS) and results from the imbalance between prooxidants and antioxidants leading to cell damage. ROS represent a variety of species, including superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\cdot}$).(48) Especially the hydroxyl radical is highly reactive, whereas superoxide and hydrogen peroxide are longer-lived. Hydroxyl radicals can be generated through the Haber-Weiss reaction, where superoxide reacts with hydrogen peroxide, or through the Fenton reaction, where hydrogen peroxide reacts with iron.

Under physiological circumstances ROS are produced by endogenous sources related to cell metabolism as a byproduct of oxygen consumption, including mitochondria, cytochrome P450 metabolism and peroxisomes.

In mitochondria, oxidative phosphorylation occurs, using oxidation of oxygen to produce adenosine triphosphate (ATP), which is the cell's most important energy source. When oxidizing oxygen, harmful intermediates like superoxides are continuously produced. The best understood producer of superoxide is coenzyme Q in complex III.(49) On average 1-2% of the oxygen used in oxidative phosphorylation results in superoxide production.

Cytochrome P450 family is a large and diverse group of enzymes, important in catalyzing the oxidation of organic substances, such as lipids, but also xenobiotics (acetaminophen) and alcohol. A superoxide radical or hydrogen peroxide can arise during the normal catalytic cycle of these enzymes.(50)

Peroxisomes harbor a variety of activities, most of which produce H_2O_2 as byproduct. In the liver, peroxisomes are particularly involved in synthesis of bile acids and breakdown of (very) long chain fatty acids through β-oxidation. The latter is especially important when the cell is low on glucose and has to turn to glycolysis for its energy.

Physiologically, reactive oxygen species also have a protective role in the liver. To combat infections, ROS are produced by immune cells through enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, myeloperoxidases present in neutrophils and monocytes or xanthine oxidase present in the serum.(51) Through these mechanisms bacteria and other pathogens can be eliminated by these reactive oxygen species.

Redox regulation

Superoxides and hydrogen peroxide were long believed to be solely toxic. However, in the past decade, many physiological roles for these reactive oxygen species have been described. (52,53) This phenomenon is also called redox signaling. For example, ROS play a role in proteolysis, especially of misfolded proteins as these are more sensitive to ROS than intact proteins.(54) Another example of redox signaling is via redox sensitive transcription factors (such as activation protein 1 or AP1 and nuclear factor kappa B or NF-κB), thereby influencing gene expression.(55) In addition, ROS is known to regulate proliferation, as proteins involved in the cell cycle were found to be redox sensitive. (56,57)

Pathological actions of reactive oxygen species

In the diseased liver, increased numbers of reactive oxygen species-producing cells, such as inflammatory cells, are present, because of the widespread inflammation. In early inflammation most oxidant stress appears to be present in the vasculature with Kupffer cells as main source.(58,59) After several hours, when the neutrophils are extravasated and adhere to their target, they have a significant and long-lasting contribution to the production of reactive oxygen species.(60) As neutrophils adhere to their target cells, ROS can enter the target cells, thereby generating intracellular oxidative stress and inducing cell death, for example in hepatocytes.

Dying hepatocytes excrete ROS as well, especially in necrosis when the cell membrane is disrupted and intracellular molecules, such as superoxide and proteases, are released in the microenvironment. Through apoptosis, hepatocytes can also enhance oxidative stress. This happens indirectly via attracting inflammatory cells through DAMPs excreted from the apoptotic bodies.

Hepatic stellate cells also produce reactive oxygen species, as NADPH oxidase (NOX) is expressed in these cells.(5)(51,61) Several NADPH oxidase isoforms have been shown to play a role in hepatic fibrosis, for example NOX1, NOX2 and NOX4.(62-64)

Furthermore, in alcoholic liver disease alcohol and its metabolites ethanol and acetaldehyde can directly generate superoxide when it is metabolized by the responsible CYP 450 enzyme (CYP2E1).(65)

Exposure of cells to reactive oxygen species can lead to severe damage, causing protein, DNA and lipid peroxidation. Products of lipid peroxidation are aldehyde-byproducts such as 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA).(66) These products are highly detrimental because of their long half-lives, allowing them to diffuse to distant intra- and extracellular targets, thereby amplifying the effects of oxidative stress. Furthermore, lipid peroxidation products are highly chemotactic (67) and may be responsible for the continuation and amplification of the injury.(68)

Also organelles can be damaged by exposure to ROS, especially mitochondria. Mitochondrial dysfunction is therefore believed to play an important role in many diseases, including liver disease. Radicals can oxidize and thereby damage for example mitochondrial DNA (MtDNA) and several mitochondrial proteins. As a consequence the mitochondrial membrane is compromised, leading to a disruption of the mitochondrial membrane potential. This is deleterious for mitochondrial function, as the mitochondrial membrane potential is a prerequisite in oxidative phosphorylation used to produce ATP. (69,70)

It is believed that oxidative stress also has a direct profibrogenic effect in hepatic stellate cells. It was shown that superoxides generated in the xanthine/xanthine oxidase system can stimulate redox-sensitive intracellular signaling resulting in increased collagen production, as well as proliferation of stellate cells.(71-73) However, we have previously shown that superoxide generated by menadione and hydrogen peroxide increase stellate cell death and inhibit proliferation.(74)

Liver disease paradox

In chronic liver disease hepatocytes die, whereas myofibroblast-like cells flourish. One hypothesis to explain this contradiction is that myofibroblasts have extra survival signals, favoring their existence over hepatocytes. We hypothesize that it is more important to contain the site of injury than trying to save damaged cells, since hepatocytes are able to proliferate and regenerate liver tissue.

A possible explanation for this paradox is that myofibroblasts are more adapted to exposure to oxidative stress, which would give them an advantage to survive in an environment where oxidative stress is excessively present.

Antioxidants

An antioxidant is defined as any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate.(48) Several cellular antioxidants exist to protect the cell from the aforementioned physiologically- or pathologically-produced reactive oxygen species.

Superoxide dismutases (SODs)

This is a family of enzymes specialized in eliminating superoxide radicals, produced either endogenously by mitochondria, or by exogenous stimuli. Three distinct SODs have been identified in mammals; Copper-zinc SOD (SOD1, or CuZnSOD), manganese SOD (SOD2 or MnSOD) and extracellular SOD (SOD3 or ECSOD).(75) Even though they are structurally unrelated and are encoded by different genes, they all elicit the same function, namely converting the superoxide radical to hydrogen peroxide.(76)

CuZnSOD (SOD1) is located in the cytosol and nucleus. Basal levels of SOD1 are higher than SOD2, but the expression of the latter is strongly inducible. SOD1 is not

vital for mice, as knockout mice develop normally into adulthood, although there are problems related to aging, for example reduced female fertility and macular degeneration. Overexpression of SOD1 does not alter expression of SOD2, and it can also not compensate for SOD2 deficiency.(77,78) A mutation in the CuZnSOD-encoding gene has been described in amyotrophic lateral sclerosis (ALS), a neurodegenerative disease affecting the motor neurons.(79)

MnSOD (SOD2) is located in the mitochondria, where most superoxides are formed. It comprises approximately 10-15% of the total SOD activity in most tissues.(80) It has a different structure and genetic organization than the other two SODs and its primary genetic structure is highly conserved in species. It has been shown to be essential for life, as knockout mice die within 2-3 weeks after birth from cardiomyopathy, metabolic acidosis and neurodegeneration.(81) In heterozygous MnSOD knockdown mice, there is a cumulative increase of oxidative DNA damage and the incidence of tumors was doubled in these mice, compared to wild type mice.(82) Research performed in the field of oncology showed that MnSOD can be considered as an important tumor suppressor protein.(83,84) MnSOD can act directly by reducing superoxide concentrations, but also indirectly through modifications in the redox state of transcription factors. Transcription factors known to be regulated by MnSOD are, among others, nuclear factor kappa B (NF-κB, important in inflammation and protection against apoptosis) and activation protein 1 (AP-1, important in proliferation). Both these transcription factors are also important in liver fibrosis as both AP-1 and NF-κB are induced in activated hepatic stellate cells.(85)

Extracellular SOD (SOD3) is a secretory protein, predominantly present in extracellular fluids and the interstitial space, and on the plasma membrane. SOD3 shows strong homology with SOD1, both on the genetic level as well as on protein level. Despite this homology, both enzymes are encoded by different genes. It is the least characterized of the three SODs.

Catalase

Catalase decomposes hydrogen peroxide (H_2O_2) to water and oxygen. It is typically localized in peroxisomes. During fatty acid beta-oxidation H_2O_2 is generated as a byproduct in peroxisomes as described above. Knockout mice for catalase develop normally and are not hypersensitive to hyperoxia-induced injury.(86) Moreover, humans suffering from the condition acatalasemia (lack of catalase) are phenotypically healthy, although a higher incidence of diabetes mellitus and progressive oral gangrene has been described.(87)

Therefore other mechanisms to decompose hydrogen peroxide must be more important or are able to compensate when catalase is unavailable.

Glutathione and glutathione peroxidases

Glutathione peroxidases (GPx) are a family of enzymes. They catalyze the reaction of glutathione (GSH) and H_2O_2 into water and oxidized glutathione (GSSG). Glutathione is highly present in almost all cells, localized in the cytosol as well as in intracellular organelles such as the endoplasmic reticulum, the nucleus and the mitochondria. It is exclusively synthesized in the cytosol in a two-step process by the rate-limiting enzyme γ-glutamylcysteine synthetase (γ-GCS or GCL) followed by γ-glutamyl transpeptidase (γ-GT).(88) Oxidized glutathione can be reduced back to GSH by the enzyme GSH reductase (GR), a reaction requiring NADPH. This possibility to recycle GSH makes it a crucial antioxidant defense mechanism for cells.

In mitochondria, GSH is mainly found in the reduced form and it represents a minor fraction of the total GSH pool (10-15%). Mitochondria lack catalase, and therefore glutathione is the most important substance in the metabolism of H_2O_2.(89)

Several glutathione peroxidases participate in the metabolism of H_2O_2, but Gpx1 is the main isoform. However, Gpx4 seems to be more important in the metabolism of H_2O_2 than Gpx1, since Gpx1 knock-out mice are fully viable, whereas Gpx4 knockout mice show embryonic lethality. Gpx4 is critical in defending the mitochondrial membrane against oxidative stress by reducing hydroperoxide groups on membrane-associated phospholipids, cholesteryl esters and lipoproteins.

Glutathione peroxidases collaborate with other peroxidase enzymes, such as glutaredoxin, thioredoxin and peroxiredoxin.(90)

Glutaredoxins (GRX) are small redox enzymes that use glutathione as a cofactor. GRX act in the antioxidant defense by reducing dehydroascorbate, peroxiredoxins, and methionine sulfoxide reductase.(91)

Thioredoxins (TRX) act as antioxidants by facilitating the reduction of other proteins by cysteine thiol-disulfide exchange. Present in all organisms, TRXs are essential for life as a loss-of-function mutation of either of the two human thioredoxin genes is lethal in an early stage of embryogenesis. The thioredoxins are kept in the reduced state by the flavoenzyme thioredoxin reductase, in a NADPH-dependent reaction. Thioredoxins act as electron donors to peroxidases and ribonucleotide reductase.(92)

Peroxiredoxins (PRX) are abundantly expressed throughout the cell. However, they have a minor contribution in the protection against oxidative stress, because of their moderate catalytic efficiency compared to glutathione peroxidase and catalase. However, next to peroxidase activity, PRXs modulate gene expression through the interaction with transcription factors. In addition, they protect against apoptosis and they play a role in innate immunity.(93)

Heme oxygenase-1

Heme oxygenases (HO's) are enzymes involved in heme degradation.(94) Heme is an indispensable cofactor in oxygen-transporting proteins (for example in hemoglobin), but also in enzymes involved in cellular respiration (cytochrome c) or drug metabolism (cytochrome P450 family).(95) To date two isoforms are known, HO-1 and HO-2. HO-1 is known as a stress-inducible protein in almost all cells (also known as heat shock protein 32 or Hsp32) whereas HO-2 is constitutively expressed.(96) There was a third isoform described, HO-3, however, this was recently shown to be a pseudogene derived from an HO-2 transcript.(97)

HO-1 is not a classical antioxidant, directly converting reactive oxygen species to less harmful molecules. However, it confers protection in multiple situations where oxidative stress is involved: e.g. in ischemic heart disease (98) and in the protection of transplanted organs via ischemic preconditioning (99-101). We have previously shown a protective effect of HO-1 against superoxide anion-induced apoptosis of hepatocytes.(102)

Protection via HO-1 is most likely conveyed through the antioxidant properties of its products: carbon monoxide (CO), biliverdin and free iron.(103) CO modulates many physiological processes, among others through its anti-inflammatory and anti-apoptotic effects. Biliverdin is converted into bilirubin, and this is able to scavenge hydroxyl radicals, singlet oxygen and superoxide anions.(44) Free iron stimulates expression of ferritin, an iron-storage protein known to have anti-oxidative properties.(104)

Natural antioxidants

Natural antioxidants such as vitamins or extracts of plants and herbs in fashion. Included in the diet or used in traditional medicine, natural products are trusted by the general public. This provides a "hot" debate and research climate, trying to find evidence for protective properties for natural antioxidants.

Vitamin E, or α-tocopherol, is known to reduce lipid peroxidation products during oxidative stress by scavenging free radicals.(105) It was shown to be protective in several *in vivo* models of liver disease, such as CCl_4-induced liver damage(106), bile duct ligation(107) and alcoholic liver disease(108). In all these studies, vitamin C was also studied, and was shown to be equally protective.

Vitamin C, or ascorbic acid, can reduce products of lipid peroxidation, and was also found to protect neutrophils from intracellularly produced reactive oxygen species (to combat microorganisms). Moreover, ascorbic acid was found to interact with hypoxia-inducible factor 1 (HIF-1), a transcription factor known to regulate many cellular processes such as growth and apoptosis, cell migration, energy metabolism etc.(109)

The precursor of vitamin A, β-carotene, acts as a highly efficient free radical scavenger, thereby protecting against lipid peroxidation.(110) Besides to this antioxidant capacity, vitamin A also elicited interest in liver fibrosis, as activated hepatic stellate cells lose their retinyl droplets within 7 days. Indeed, the outdated term for stellate cells used to be vitamin A-storing cells. Vitamin A is also important to repress inflammation.

Despite the above mentioned studies, there is not enough evidence to date to use vitamins neither in a therapeutic setting nor in preventive medicine, as long as there is a balanced and complete diet.(111,112)

Nowadays, many plant and herbal extracts are studied for their potential antioxidative properties. One of the most investigated compounds is curcumin.(113) For centuries curcumin has been used in traditional medicine, for a wide range of diseases, including liver disease. Many *in vitro* studies showed anti-inflammatory and antioxidative effects of this compound. However, in clinical studies the evidence is limited, especially due to poor absorption of curcumin following oral administration.

Another widely used product, coffee, emerges as a protective agent in liver disease. Several epidemiological studies have shown that coffee consumption reduces progression of liver disease, but also is able to prevent liver disease.(114-116) As there are many components in coffee, it is unknown which component is responsible for the protective effect. It is speculated that caffeine plays an important role, as there are also studies showing that caffeine intake is protective.(117-119) Several *in vivo* animal studies, focusing on different models of liver disease, have confirmed these epidemiological data.(120-124) However, the molecular mechanisms are not yet known.

Current therapeutic options

Currently, there are no curative options for liver fibrosis or cirrhosis, apart from liver transplantation and/or removal of the harmful stimulus. Very effective treatment regimens have been developed for hepatitis B and C, achieving a sustained virologic response, which is correlated with decreased incidence of full-blown liver cirrhosis and its symptoms.(125,126)

Anti-inflammatory agents, such as corticosteroids and immunosuppressants (i.e. cyclosporine A and tacrolimus), are used to inhibit progression of liver fibrosis, especially in auto-immune hepatitis. However, these therapies are accompanied by many side effects. Moreover, corticosteroids do not completely suppress progression of the disease, and there is no evidence for the use of immunosuppressants.

The ideal therapy for chronic liver disease would be to target fibrogenesis, through specific targeting of extracellular matrix-producing myofibroblasts. Several drugs were tested in randomized controlled trials, for example interferon-gamma or peroxisome proliferator-activated receptor-gamma (PPAR-γ) agonists (thiazolidinedione). However, the disease progression rate was not reduced by long-term use of interferon-gamma.(127,128) PPAR-γ agonists seemed very promising in *in vitro* and *in vivo* models, and also in initial patient trials. However, none of these randomized controlled trials revealed long-term efficacy.(129)

Scope of the thesis

The aim of this thesis is to investigate the mechanism(s) of hepatic stellate cell activation focusing on the role of oxidative stress and anti-oxidant mechanisms, in order to develop a stellate cell-targeted therapy for liver fibrosis.

In **chapter 2**, we investigated the role of heme oxygenase-1 in cells most intimately involved in liver fibrogenesis, hepatic stellate cells and macrophages.

Cells can deal with reactive oxygen species by converting superoxide into hydrogen peroxide using superoxide dismutases. Subsequently, hydrogen peroxide can be converted into water and oxygen by catalase and glutathione peroxidase. In **chapter 3**, we investigated the role of antioxidant systems in the resistance of activated hepatic stellate cells against hydrogen peroxide-induced toxicity. In **chapter 4**, we studied the role of mitochondrial manganese superoxide dismutase (MnSOD) in hepatic stellate cells and we investigated the feasibility of reversing the phenotype of activated hepatic stellate cells to a quiescent phenotype by inducing MnSOD levels.

As caffeine seems to have antifibrotic properties, we investigated the effect of caffeine *in vitro* on hepatic stellate cells, portal myofibroblasts, macrophages and hepatocytes in **chapter 5**.

Finally, in **chapter 6**, the results from this thesis will be discussed, focusing on the role of adaptive mechanisms of hepatic stellate cells in liver fibrogenesis and how to use this knowledge to develop therapies for liver fibrosis.

References

(1) CBS S. Doodsoorzaken; korte lijst (belangrijke doodsoorzaken), leeftijd, geslacht. 2012; Available at: http://statline.cbs.nl. Accessed 07/31, 2013.

(2) Goldberg E, Chopra S. Overview of the complications, prognosis, and management of cirrhosis. 2013; Available at: http://www.uptodate.com. Accessed 07/31, 2013.

(3) Younossi ZM, Stepanova M, Negro F, Hallaji S, Younossi Y, Lam B, et al. Nonalcoholic fatty liver disease in lean individuals in the United States. Medicine (Baltimore) 2012 Nov;91(6):319-327.

(4) Geerts A. History, heterogeneity, developmental biology, and functions of quiescent hepatic stellate cells. Semin Liver Dis 2001 Aug;21(3):311-335.

(5) Brenner DA. Molecular pathogenesis of liver fibrosis. Trans Am Clin Climatol Assoc 2009;120:361-368.

(6) Friedman SL. Mechanisms of hepatic fibrogenesis. Gastroenterology 2008 May;134(6):1655-1669.

(7) She H, Xiong S, Hazra S, Tsukamoto H. Adipogenic transcriptional regulation of hepatic stellate cells. J Biol Chem 2005 Feb 11;280(6):4959-4967.

(8) Chen A, Tang Y, Davis V, Hsu FF, Kennedy SM, Song H, et al. Liver fatty acid binding protein (L-Fabp) modulates murine stellate cell activation and diet-induced nonalcoholic fatty liver disease. Hepatology 2013 Jun;57(6):2202-2212.

(9) Chen Y, Choi SS, Michelotti GA, Chan IS, Swiderska-Syn M, Karaca GF, et al. Hedgehog controls hepatic stellate cell fate by regulating metabolism. Gastroenterology 2012 Nov;143(5):1319-29.e1-11.

(10) Seki E, De Minicis S, Osterreicher CH, Kluwe J, Osawa Y, Brenner DA, et al. TLR4 enhances TGF-beta signaling and hepatic fibrosis. Nat Med 2007 Nov;13(11):1324-1332.

(11) Canbay A, Taimr P, Torok N, Higuchi H, Friedman S, Gores GJ. Apoptotic body engulfment by a human stellate cell line is profibrogenic. Lab Invest 2003 May;83(5):655-663.

(12) Jiang JX, Mikami K, Venugopal S, Li Y, Torok NJ. Apoptotic body engulfment by hepatic stellate cells promotes their survival by the JAK/STAT and Akt/NF-kappaB-dependent pathways. J Hepatol 2009 Jul;51(1):139-148.

(13) Hazra S, Xiong S, Wang J, Rippe RA, Krishna V, Chatterjee K, et al. Peroxisome proliferator-activated receptor gamma induces a phenotypic switch from activated to quiescent hepatic stellate cells. J Biol Chem 2004 Mar 19;279(12):11392-11401.

(14) Dranoff JA, Wells RG. Portal fibroblasts: Underappreciated mediators of biliary fibrosis. Hepatology 2010 Apr;51(4):1438-1444.

(15) Tuchweber B, Desmouliere A, Bochaton-Piallat ML, Rubbia-Brandt L, Gabbiani G. Proliferation and phenotypic modulation of portal fibroblasts in the early stages of cholestatic fibrosis in the rat. Lab Invest 1996 Jan;74(1):265-278.

(16) Beaussier M, Wendum D, Schiffer E, Dumont S, Rey C, Lienhart A, et al. Prominent contribution of portal mesenchymal cells to liver fibrosis in ischemic and obstructive cholestatic injuries. Lab Invest 2007 Mar;87(3):292-303.

(17) Bosselut N, Housset C, Marcelo P, Rey C, Burmester T, Vinh J, et al. Distinct proteomic features of two fibrogenic liver cell populations: hepatic stellate cells and portal myofibroblasts. Proteomics 2010 Mar;10(5):1017-1028.

(18) Wells RG. Cellular sources of extracellular matrix in hepatic fibrosis. Clin Liver Dis 2008 Nov;12(4):759-68, viii.

(19) Novo E, di Bonzo LV, Cannito S, Colombatto S, Parola M. Hepatic myofibroblasts: a heterogeneous population of multifunctional cells in liver fibrogenesis. Int J Biochem Cell Biol 2009 Nov;41(11):2089-2093.

(20) Kisseleva T, Uchinami H, Feirt N, Quintana-Bustamante O, Segovia JC, Schwabe RF, et al. Bone marrow-derived fibrocytes participate in pathogenesis of liver fibrosis. J Hepatol 2006 Sep;45(3):429-438.

(21) Taura K, Miura K, Iwaisako K, Osterreicher CH, Kodama Y, Penz-Osterreicher M, et al. Hepatocytes do not undergo epithelial-mesenchymal transition in liver fibrosis in mice. Hepatology 2010 Mar;51(3):1027-1036.

(22) Chu AS, Diaz R, Hui JJ, Yanger K, Zong Y, Alpini G, et al. Lineage tracing demonstrates no evidence of cholangiocyte epithelial-to-mesenchymal transition in murine models of hepatic fibrosis. Hepatology 2011 May;53(5):1685-1695.

(23) Poli G. Pathogenesis of liver fibrosis: role of oxidative stress. Mol Aspects Med 2000 Jun;21(3):49-98.

(24) Seyer JM. Interstitial collagen polymorphism in rat liver with CCl4-induced cirrhosis. Biochim Biophys Acta 1980 May 22;629(3):490-498.

(25) Sato S, Adachi A, Wakamatsu K, Sasaki Y, Satomura K, Asano G. Abnormal elastic system fibers in fibrotic human liver. Med Electron Microsc 2000;33(3):135-142.

(26) George J, Tsutsumi M, Takase S. Expression of hyaluronic acid in N-nitrosodimethylamine induced hepatic fibrosis in rats. Int J Biochem Cell Biol 2004 Feb;36(2):307-319.

(27) Pellicoro A, Ramachandran P, Iredale JP. Reversibility of liver fibrosis. Fibrogenesis Tissue Repair 2012 Jun 6;5 Suppl 1:S26.

(28) Kossakowska AE, Edwards DR, Lee SS, Urbanski LS, Stabbler AL, Zhang CL, et al. Altered balance between matrix metalloproteinases and their inhibitors in experimental biliary fibrosis. Am J Pathol 1998 Dec;153(6):1895-1902.

(29) Park SY, Shin HW, Lee KB, Lee MJ, Jang JJ. Differential expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in thioacetamide-induced chronic liver injury. J Korean Med Sci 2010 Apr;25(4):570-576.

(30) Wanless IR, Nakashima E, Sherman M. Regression of human cirrhosis. Morphologic features and the genesis of incomplete septal cirrhosis. Arch Pathol Lab Med 2000 Nov;124(11):1599-1607.

(31) Pan Q, Zhang ZB, Zhang X, Shi J, Chen YX, Han ZG, et al. Gene expression profile analysis of the spontaneous reversal of rat hepatic fibrosis by cDNA microarray. Dig Dis Sci 2007 Oct;52(10):2591-2600.

(32) Kisseleva T, Cong M, Paik Y, Scholten D, Jiang C, Benner C, et al. Myofibroblasts revert to an inactive phenotype during regression of liver fibrosis. Proc Natl Acad Sci U S A 2012 Jun 12;109(24):9448-9453.

(33) Hartland SN, Murphy F, Aucott RL, Abergel A, Zhou X, Waung J, et al. Active matrix metalloproteinase-2 promotes apoptosis of hepatic stellate cells via the cleavage of cellular N-cadherin. Liver Int 2009 Aug;29(7):966-978.

(34) Pellicoro A, Aucott RL, Ramachandran P, Robson AJ, Fallowfield JA, Snowdon VK, et al. Elastin accumulation is regulated at the level of degradation by macrophage metalloelastase (MMP-12) during experimental liver fibrosis. Hepatology 2012 Jun;55(6):1965-1975.

(35) Fallowfield JA, Mizuno M, Kendall TJ, Constandinou CM, Benyon RC, Duffield JS, et al. Scar-associated macrophages are a major source of hepatic matrix metalloproteinase-13 and facilitate the resolution of murine hepatic fibrosis. J Immunol 2007 Apr 15;178(8):5288-5295.

(36) Issa R, Zhou X, Constandinou CM, Fallowfield J, Millward-Sadler H, Gaca MD, et al. Spontaneous recovery from micronodular cirrhosis: evidence for incomplete resolution associated with matrix cross-linking. Gastroenterology 2004 Jun;126(7):1795-1808.

(37) Kubes P, Mehal WZ. Sterile inflammation in the liver. Gastroenterology 2012 Nov;143(5):1158-1172.

(38) Rock KL, Latz E, Ontiveros F, Kono H. The sterile inflammatory response. Annu Rev Immunol 2010;28:321-342.

(39) McDonald B, Pittman K, Menezes GB, Hirota SA, Slaba I, Waterhouse CC, et al. Intravascular danger signals guide neutrophils to sites of sterile inflammation. Science 2010 Oct 15;330(6002):362-366.

(40) Saito JM, Bostick MK, Campe CB, Xu J, Maher JJ. Infiltrating neutrophils in bile duct-ligated livers do not promote hepatic fibrosis. Hepatol Res 2003 Feb;25(2):180-191.

(41) Wynn TA, Barron L, Thompson RW, Madala SK, Wilson MS, Cheever AW, et al. Quantitative assessment of macrophage functions in repair and fibrosis. Curr Protoc Immunol 2011 Apr;Chapter 14:Unit14.22.

(42) Kono H, Karmarkar D, Iwakura Y, Rock KL. Identification of the cellular sensor that stimulates the inflammatory response to sterile cell death. J Immunol 2010 Apr 15;184(8):4470-4478.

(43) Tacke F. Functional role of intrahepatic monocyte subsets for the progression of liver inflammation and liver fibrosis in vivo. Fibrogenesis Tissue Repair 2012 Jun 6;5 Suppl 1:S27.

(44) Dudnik LB, Khrapova NG. Characterization of bilirubin inhibitory properties in free radical oxidation reactions. Membr Cell Biol 1998;12(2):233-240.

(45) Duffield JS, Forbes SJ, Constandinou CM, Clay S, Partolina M, Vuthoori S, et al. Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. J Clin Invest 2005 Jan;115(1):56-65.

(46) Mitchell C, Couton D, Couty JP, Anson M, Crain AM, Bizet V, et al. Dual role of CCR2 in the constitution and the resolution of liver fibrosis in mice. Am J Pathol 2009 May;174(5):1766-1775.

(47) Wynn TA, Cheever AW, Jankovic D, Poindexter RW, Caspar P, Lewis FA, et al. An IL-12-based vaccination method for preventing fibrosis induced by schistosome infection. Nature 1995 Aug 17;376(6541):594-596.
(48) Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. 2nd edition ed. Oxford and New York: Clarendon Press; 1989.
(49) Jezek P, Hlavata L. Mitochondria in homeostasis of reactive oxygen species in cell, tissues, and organism. Int J Biochem Cell Biol 2005 Dec;37(12):2478-2503.
(50) Loida PJ, Sligar SG. Molecular recognition in cytochrome P-450: mechanism for the control of uncoupling reactions. Biochemistry 1993 Nov 2;32(43):11530-11538.
(51) Muriel P. Role of free radicals in liver diseases. Hepatol Int 2009 Nov 26.
(52) Mittler R, Vanderauwera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, et al. ROS signaling: the new wave? Trends Plant Sci 2011 Jun;16(6):300-309.
(53) Buetler TM, Krauskopf A, Ruegg UT. Role of superoxide as a signaling molecule. News Physiol Sci 2004 Jun;19:120-123.
(54) Dukan S, Farewell A, Ballesteros M, Taddei F, Radman M, Nystrom T. Protein oxidation in response to increased transcriptional or translational errors. Proc Natl Acad Sci U S A 2000 May 23;97(11):5746-5749.
(55) Arrigo AP. Gene expression and the thiol redox state. Free Radic Biol Med 1999 Nov;27(9-10):936-944.
(56) Oberley TD, Schultz JL, Li N, Oberley LW. Antioxidant enzyme levels as a function of growth state in cell culture. Free Radic Biol Med 1995 Jul;19(1):53-65.
(57) Kwon YW, Masutani H, Nakamura H, Ishii Y, Yodoi J. Redox regulation of cell growth and cell death. Biol Chem 2003 Jul;384(7):991-996.
(58) Jaeschke H, Bautista AP, Spolarics Z, Spitzer JJ. Superoxide generation by Kupffer cells and priming of neutrophils during reperfusion after hepatic ischemia. Free Radic Res Commun 1991;15(5):277-284.
(59) Bautista AP. Acute ethanol binge followed by withdrawal regulates production of reactive oxygen species and cytokine-induced neutrophil chemoattractant and liver injury during reperfusion after hepatic ischemia. Antioxid Redox Signal 2002 Oct;4(5):721-731.
(60) Jaeschke H, Smith CW. Mechanisms of neutrophil-induced parenchymal cell injury. J Leukoc Biol 1997 Jun;61(6):647-653.
(61) Paik YH, Kim J, Aoyama T, De Minicis S, Bataller R, Brenner DA. Role of NADPH Oxidases in Liver Fibrosis. Antioxid Redox Signal 2013 Sep 16.
(62) Cui W, Matsuno K, Iwata K, Ibi M, Matsumoto M, Zhang J, et al. NOX1/nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) oxidase promotes proliferation of stellate cells and aggravates liver fibrosis induced by bile duct ligation. Hepatology 2011 Sep 2;54(3):949-958.
(63) Sancho P, Mainez J, Crosas-Molist E, Roncero C, Fernandez-Rodriguez CM, Pinedo F, et al. NADPH oxidase NOX4 mediates stellate cell activation and hepatocyte cell death during liver fibrosis development. PLoS One 2012;7(9):e45285.
(64) Paik YH, Iwaisako K, Seki E, Inokuchi S, Schnabl B, Osterreicher CH, et al. The nicotinamide adenine dinucleotide phosphate oxidase (NOX) homologues NOX1 and NOX2/gp91(phox) mediate hepatic fibrosis in mice. Hepatology 2011 May;53(5):1730-1741.
(65) Ekstrom G, Ingelman-Sundberg M. Rat liver microsomal NADPH-supported oxidase activity and lipid peroxidation dependent on ethanol-inducible cytochrome P-450 (P-450IIE1). Biochem Pharmacol 1989 Apr 15;38(8):1313-1319.
(66) Perluigi M, Coccia R, Butterfield DA. 4-Hydroxy-2-nonenal, a reactive product of lipid peroxidation, and neurodegenerative diseases: a toxic combination illuminated by redox proteomics studies. Antioxid Redox Signal 2012 Dec 1;17(11):1590-1609.
(67) Curzio M, Esterbauer H, Di Mauro C, Cecchini G, Dianzani MU. Chemotactic activity of the lipid peroxidation product 4-hydroxynonenal and homologous hydroxyalkenals. Biol Chem Hoppe Seyler 1986 Apr;367(4):321-329.
(68) Liu P, Vonderfecht SL, McGuire GM, Fisher MA, Farhood A, Jaeschke H. The 21-aminosteroid tirilazad mesylate protects against endotoxin shock and acute liver failure in rats. J Pharmacol Exp Ther 1994 Oct;271(1):438-445.
(69) Nunnari J, Suomalainen A. Mitochondria: in sickness and in health. Cell 2012 Mar 16;148(6):1145-1159.
(70) Zavodnik IB, Dremza IK, Cheshchevik VT, Lapshina EA, Zamaraewa M. Oxidative damage of rat liver mitochondria during exposure to t-butyl hydroperoxide. Role of Ca(2)(+) ions in oxidative processes. Life Sci 2013 Jun 21;92(23):1110-1117.

(71) Casini A, Ceni E, Salzano R, Biondi P, Parola M, Galli A, et al. Neutrophil-derived superoxide anion induces lipid peroxidation and stimulates collagen synthesis in human hepatic stellate cells: role of nitric oxide. Hepatology 1997 Feb;25(2):361-367.

(72) Moshage H, Casini A, Lieber CS. Acetaldehyde selectively stimulates collagen production in cultured rat liver fat-storing cells but not in hepatocytes. Hepatology 1990 Sep;12(3 Pt 1):511-518.

(73) Galli A, Svegliati-Baroni G, Ceni E, Milani S, Ridolfi F, Salzano R, et al. Oxidative stress stimulates proliferation and invasiveness of hepatic stellate cells via a MMP2-mediated mechanism. Hepatology 2005 May;41(5):1074-1084.

(74) Dunning S, Hannivoort RA, de Boer JF, Buist-Homan M, Faber KN, Moshage H. Superoxide anions and hydrogen peroxide inhibit proliferation of activated rat stellate cells and induce different modes of cell death. Liver Int 2009 Jul;29(6):922-932.

(75) Miao L, St Clair DK. Regulation of superoxide dismutase genes: implications in disease. Free Radic Biol Med 2009 Aug 15;47(4):344-356.

(76) Fridovich I. Superoxide radical and superoxide dismutases. Annu Rev Biochem 1995;64:97-112.

(77) White CW, Nguyen DH, Suzuki K, Taniguchi N, Rusakow LS, Avraham KB, et al. Expression of manganese superoxide dismutase is not altered in transgenic mice with elevated level of copper-zinc superoxide dismutase. Free Radic Biol Med 1993 Dec;15(6):629-636.

(78) Copin JC, Gasche Y, Chan PH. Overexpression of copper/zinc superoxide dismutase does not prevent neonatal lethality in mutant mice that lack manganese superoxide dismutase. Free Radic Biol Med 2000 May 15;28(10):1571-1576.

(79) Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, et al. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 1993 Mar 4;362(6415):59-62.

(80) Tsan MF. Superoxide dismutase and pulmonary oxygen toxicity: lessons from transgenic and knockout mice (Review). Int J Mol Med 2001 Jan;7(1):13-19.

(81) Li Y, Huang TT, Carlson EJ, Melov S, Ursell PC, Olson JL, et al. Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. Nat Genet 1995 Dec;11(4):376-381.

(82) Van Remmen H, Ikeno Y, Hamilton M, Pahlavani M, Wolf N, Thorpe SR, et al. Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging. Physiol Genomics 2003 Dec 16;16(1):29-37.

(83) Kinnula VL, Crapo JD. Superoxide dismutases in malignant cells and human tumors. Free Radic Biol Med 2004 Mar 15;36(6):718-744.

(84) Holley AK, Dhar SK, Xu Y, St Clair DK. Manganese superoxide dismutase: beyond life and death. Amino Acids 2012 Jan;42(1):139-158.

(85) Mann DA, Smart DE. Transcriptional regulation of hepatic stellate cell activation. Gut 2002 Jun;50(6):891-896.

(86) Ho YS, Xiong Y, Ma W, Spector A, Ho DS. Mice lacking catalase develop normally but show differential sensitivity to oxidant tissue injury. J Biol Chem 2004 Jul 30;279(31):32804-32812.

(87) Goth L, Rass P, Pay A. Catalase enzyme mutations and their association with diseases. Mol Diagn 2004;8(3):141-149.

(88) Rahman I, MacNee W. Oxidative stress and regulation of glutathione in lung inflammation. Eur Respir J 2000 Sep;16(3):534-554.

(89) Mari M, Morales A, Colell A, Garcia-Ruiz C, Fernandez-Checa JC. Mitochondrial glutathione, a key survival antioxidant. Antioxid Redox Signal 2009 Nov;11(11):2685-2700.

(90) Hanschmann EM, Godoy JR, Berndt C, Hudemann C, Lillig CH. Thioredoxins, Glutaredoxins, and Peroxiredoxins-Molecular Mechanisms and Health Significance: from Cofactors to Antioxidants to Redox Signaling. Antioxid Redox Signal 2013 Mar 28.

(91) Lillig CH, Berndt C. Glutaredoxins in thiol/disulfide exchange. Antioxid Redox Signal 2013 May 1;18(13):1654-1665.

(92) Mahmood DF, Abderrazak A, Khadija EH, Simmet T, Rouis M. The Thioredoxin System as a Therapeutic Target in Human Health and Disease. Antioxid Redox Signal 2013 Feb 26.

(93) Ishii T, Warabi E, Yanagawa T. Novel roles of peroxiredoxins in inflammation, cancer and innate immunity. J Clin Biochem Nutr 2012 Mar;50(2):91-105.

(94) Sass G, Barikbin R, Tiegs G. The multiple functions of heme oxygenase-1 in the liver. Z Gastroenterol 2012 Jan;50(1):34-40.
(95) Grochot-Przeczek A, Dulak J, Jozkowicz A. Haem oxygenase-1: non-canonical roles in physiology and pathology. Clin Sci (Lond) 2012 Feb;122(3):93-103.
(96) Braggins PE, Trakshel GM, Kutty RK, Maines MD. Characterization of two heme oxygenase isoforms in rat spleen: comparison with the hematin-induced and constitutive isoforms of the liver. Biochem Biophys Res Commun 1986 Dec 15;141(2):528-533.
(97) Hayashi S, Omata Y, Sakamoto H, Higashimoto Y, Hara T, Sagara Y, et al. Characterization of rat heme oxygenase-3 gene. Implication of processed pseudogenes derived from heme oxygenase-2 gene. Gene 2004 Jul 21;336(2):241-250.
(98) Zeng B, Lin G, Ren X, Zhang Y, Chen H. Over-expression of HO-1 on mesenchymal stem cells promotes angiogenesis and improves myocardial function in infarcted myocardium. J Biomed Sci 2010 Oct 7;17:80.
(99) Lai IR, Chang KJ, Chen CF, Tsai HW. Transient limb ischemia induces remote preconditioning in liver among rats: the protective role of heme oxygenase-1. Transplantation 2006 05/15;81(0041-1337; 9):1311-1317.
(100) Jaeschke H. Molecular mechanisms of hepatic ischemia-reperfusion injury and preconditioning. Am J Physiol Gastrointest Liver Physiol 2003 Jan;284(1):G15-26.
(101) Redaelli CA, Tian YH, Schaffner T, Ledermann M, Baer HU, Dufour JF. Extended preservation of rat liver graft by induction of heme oxygenase-1. Hepatology 2002 May;35(5):1082-1092.
(102) Conde de la Rosa L, Vrenken TE, Hannivoort RA, Buist-Homan M, Havinga R, Slebos DJ, et al. Carbon monoxide blocks oxidative stress-induced hepatocyte apoptosis via inhibition of the p54 JNK isoform. Free Radic Biol Med 2008 Apr 1;44(7):1323-1333.
(103) Rochette L, Cottin Y, Zeller M, Vergely C. Carbon monoxide: mechanisms of action and potential clinical implications. Pharmacol Ther 2013 Feb;137(2):133-152.
(104) Arosio P, Ingrassia R, Cavadini P. Ferritins: a family of molecules for iron storage, antioxidation and more. Biochim Biophys Acta 2009 Jul;1790(7):589-599.
(105) Traber MG, Atkinson J. Vitamin E, antioxidant and nothing more. Free Radic Biol Med 2007 Jul 1;43(1):4-15.
(106) Kataoka T, Nishiyama Y, Yamato K, Teraoka J, Morii Y, Sakoda A, et al. Comparative study on the inhibitory effects of antioxidant vitamins and radon on carbon tetrachloride-induced hepatopathy. J Radiat Res 2012 Nov 1;53(6):830-839.
(107) Soylu AR, Aydogdu N, Basaran UN, Altaner S, Tarcin O, Gedik N, et al. Antioxidants vitamin E and C attenuate hepatic fibrosis in biliary-obstructed rats. World J Gastroenterol 2006 Nov 14;12(42):6835-6841.
(108) Prathibha P, Rejitha S, Harikrishnan R, Das SS, Abhilash PA, Indira M. Additive effect of alpha-tocopherol and ascorbic acid in combating ethanol-induced hepatic fibrosis. Redox Rep 2013;18(1):36-46.
(109) Traber MG, Stevens JF. Vitamins C and E: beneficial effects from a mechanistic perspective. Free Radic Biol Med 2011 Sep 1;51(5):1000-1013.
(110) Stahl W, Sies H. Antioxidant activity of carotenoids. Mol Aspects Med 2003 Dec;24(6):345-351.
(111) Bjelakovic G, Gluud LL, Nikolova D, Bjelakovic M, Nagorni A, Gluud C. Antioxidant supplements for liver diseases. Cochrane Database Syst Rev 2011 Mar 16;(3):CD007749. doi(3):CD007749.
(112) Singal AK, Jampana SC, Weinman SA. Antioxidants as therapeutic agents for liver disease. Liver Int 2011 Nov;31(10):1432-1448.
(113) Vera-Ramirez L, Perez-Lopez P, Varela-Lopez A, Ramirez-Tortosa M, Battino M, Quiles JL. Curcumin and liver disease. Biofactors 2013 Jan 10.
(114) Freedman ND, Curto TM, Lindsay KL, Wright EC, Sinha R, Everhart JE, et al. Coffee consumption is associated with response to peginterferon and ribavirin therapy in patients with chronic hepatitis C. Gastroenterology 2011 Jun;140(7):1961-1969.
(115) Freedman ND, Everhart JE, Lindsay KL, Ghany MG, Curto TM, Shiffman ML, et al. Coffee intake is associated with lower rates of liver disease progression in chronic hepatitis C. Hepatology 2009 Nov;50(5):1360-1369.
(116) Sang LX, Chang B, Li XH, Jiang M. Consumption of coffee associated with reduced risk of liver cancer: a meta-analysis. BMC Gastroenterol 2013 Feb 25;13:34-230X-13-34.
(117) Modi AA, Feld JJ, Park Y, Kleiner DE, Everhart JE, Liang TJ, et al. Increased caffeine consumption is associated with reduced hepatic fibrosis. Hepatology 2010 Jan;51(1):201-209.

(118) Molloy JW, Calcagno CJ, Williams CD, Jones FJ, Torres DM, Harrison SA. Association of coffee and caffeine consumption with fatty liver disease, nonalcoholic steatohepatitis, and degree of hepatic fibrosis. Hepatology 2012 Feb;55(2):429-436.

(119) Birerdinc A, Stepanova M, Pawloski L, Younossi ZM. Caffeine is protective in patients with non-alcoholic fatty liver disease. Aliment Pharmacol Ther 2012 Jan;35(1):76-82.

(120) Klemmer I, Yagi S, Gressner OA. Oral application of 1,7-dimethylxanthine (paraxanthine) attenuates the formation of experimental cholestatic liver fibrosis. Hepatol Res 2011 Nov;41(11):1094-1109.

(121) Moreno MG, Chavez E, Aldaba-Muruato LR, Segovia J, Vergara P, Tsutsumi V, et al. Coffee prevents CCl(4)-induced liver cirrhosis in the rat. Hepatol Int 2011 Sep;5(3):857-863.

(122) Furtado KS, Prado MG, Aguiar E Silva MA, Dias MC, Rivelli DP, Rodrigues MA, et al. Coffee and caffeine protect against liver injury induced by thioacetamide in male Wistar rats. Basic Clin Pharmacol Toxicol 2012 Nov;111(5):339-347.

(123) Yamauchi R, Kobayashi M, Matsuda Y, Ojika M, Shigeoka S, Yamamoto Y, et al. Coffee and caffeine ameliorate hyperglycemia, fatty liver, and inflammatory adipocytokine expression in spontaneously diabetic KK-Ay mice. J Agric Food Chem 2010 May 12;58(9):5597-5603.

(124) Lv X, Chen Z, Li J, Zhang L, Liu H, Huang C, et al. Caffeine protects against alcoholic liver injury by attenuating inflammatory response and oxidative stress. Inflamm Res 2010 Aug;59(8):635-645.

(125) Liang TJ, Ghany MG. Current and future therapies for hepatitis C virus infection. N Engl J Med 2013 May 16;368(20):1907-1917.

(126) Huang R, Hao Y, Zhang J, Wu C. Interferon-alpha plus adefovir combination therapy versus interferon-alpha monotherapy for chronic hepatitis B treatment: A meta-analysis. Hepatol Res 2013 Jan 10.

(127) Di Bisceglie AM, Shiffman ML, Everson GT, Lindsay KL, Everhart JE, Wright EC, et al. Prolonged therapy of advanced chronic hepatitis C with low-dose peginterferon. N Engl J Med 2008 Dec 4;359(23):2429-2441.

(128) Pockros PJ, Jeffers L, Afdhal N, Goodman ZD, Nelson D, Gish RG, et al. Final results of a double-blind, placebo-controlled trial of the antifibrotic efficacy of interferon-gamma1b in chronic hepatitis C patients with advanced fibrosis or cirrhosis. Hepatology 2007 Mar;45(3):569-578.

(129) Zhang F, Kong D, Lu Y, Zheng S. Peroxisome proliferator-activated receptor-gamma as a therapeutic target for hepatic fibrosis: from bench to bedside. Cell Mol Life Sci 2013 Jan;70(2):259-276.

Chapter 2

Heme oxygenase-1 has an immunomodulatory role in liver fibrosis

Marjolein H Tiebosch[1], Manon Buist-Homan[2], Anton Tiebosch[3],
Klaas Nico Faber[1], Han Moshage[1]

[1]Dept. Gastroenterology and Hepatology, [2]Dept. Laboratory Medicine, University Medical Center Groningen, University of Groningen, Groningen and [3]Dept. Pathology, Martini Hospital, Groningen, The Netherlands

Submitted

Abstract

Background Liver fibrosis is caused by a continuous wound healing response accompanied by inflammation and oxidative stress. Quiescent hepatic stellate cells (HSCs) transdifferentiate in activated myofibroblast-like cells to produce extracellular matrix. Oxidative stress induces heme oxygenase-1 (HO-1), which is believed to have an antifibrogenic but also an immunomodulatory role.

Aims This study aimed to investigate the cellular distribution of HO-1 during liver fibrogenesis *in vivo* and to investigate the role of HO-1 in the inflammatory and fibrotic response *in vitro*.

Methods Bile duct ligation (BDL, 4 days to 4 weeks) was used as an *in vivo* model of liver damage; HO-1, αSMA and ED-1 expression were determined using immunohistochemistry. For *in vitro* experiments, culture-activated rat HSCs, primary rat Kupffer cells and the mouse macrophage cell line Raw 264.7 were used. HO-1 induction was achieved by infection with an adenovirus expressing HO-1 or using hemin. Expression of M1 and M2 type macrophage markers and HSC activation markers were determined using qPCR.

Results *In vivo*, HO-1 expression was only localized in macrophage-like cells upon BDL. In cultured macrophages, HO-1 induction induced M1 type macrophage-related genes and reduced genes associated with M2 type macrophage. Induction of HO-1 did not change αSMA nor collagen type 1 mRNA expression in activated HSCs.

Conclusions HO-1 is predominantly expressed in macrophages and an induction of HO-1 causes a shift to the anti-fibrotic M1 phenotype. Thus, in liver fibrogenesis HO-1 seems to have an indirect protective effect via its immunomodulatory properties, instead of a direct antifibrogenic effect.

Introduction

The incidence of chronic liver diseases evolving into liver fibrosis and cirrhosis is increasing worldwide. This is caused to a great extent by the increased incidence of obesity and accompanying liver disorders like non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) that evolve into liver fibrosis.(1,2) Liver fibrosis and its end stage liver cirrhosis are severe disorders with a high morbidity and mortality.

Currently, no effective drug-based treatment is available to reverse or arrest fibrosis and/or cirrhosis. Therefore, there is an urgent need to develop novel therapies to effectively treat patients with liver fibrosis.

Liver fibrosis is characterized by increased production and deposition of extracellular matrix (ECM) by myofibroblast-like cells from different origins in the liver, such as the hepatic stellate cells (HSCs) and the portal myofibroblasts (PMFs). During fibrogenesis these cells transdifferentiate from a quiescent to an activated phenotype, with high expression of collagen type I and α-Smooth Muscle Actin (α-SMA).

The excessive production of the ECM by transdifferentiated myofibroblast-like cells is the result of a damaging stimulus to the liver leading to a continuous wound healing response. Inflammatory mediators are released by damaged cells and inflammatory cells, perpetuating the wound healing response. Inflammatory cells that play an important role in liver fibrosis are the resident macrophages, the Kupffer cells, but also macrophages recruited from the bone marrow.

Most chronic liver diseases are accompanied by excessive production of reactive oxygen species (ROS) in the injured liver. These ROS are predominantly released in the inflammatory reaction. In contrast to hepatocytes, which perish in this hostile environment, HSCs and PMFs become activated and proliferate. This paradox may be explained by a resistance to ROS-induced toxicity. Indeed, it has been speculated that ROS may in fact promote stellate cell activation and proliferation.(3-9)

In this respect, the enzyme heme oxygenase-1 (HO-1) has elicited much interest. HO-1 is the inducible form of the rate-limiting enzyme in heme degradation. Its products, carbon monoxide and biliverdin, have anti-oxidant properties.(10) HO-1 is strongly induced upon oxidative stress and is involved in the protection against oxidative stress-induced damage in many organs: e.g. in ischemic heart disease (11), tubule-interstitial fibrosis in the kidney (12) and pulmonary fibrosis (13). In cancer, HO-1 plays an anti-apoptotic role and is therefore associated with adverse outcomes.(14) A protective effect

of HO-1 is also observed in the liver and HO-1 is (in part) responsible for the phenomenon of ischemic preconditioning in which organs become increasingly resistant to oxidative stress after a prior, minor exposure to oxidative stress.(15-17) We have previously shown that overexpression of HO-1 in hepatocytes increases the resistance of hepatocytes to superoxide anion-induced apoptotic cell death.(18) In this study we also showed an induction of HO-1 mRNA and protein expression in a bile duct ligation model.

HO-1 induction can retard or even reverse fibrosis in several organs, such as lung and kidney.(12,13) However, concerning liver fibrosis there are contradictory reports with regard to the antifibrotic effect of HO-1. A direct antifibrogenic effect of HO-1 has been proposed in human myofibroblasts isolated from human fibrotic liver explants (19,20). Alternatively, HO-1 is known for its immunomodulatory role, as induction of HO-1 has been shown to be protective in multiple inflammation models.(21,22) Therefore, an indirect role of HO-1, modulating liver fibrosis by suppressing inflammatory responses in the liver, is plausible.

The aims of this study were to investigate the cellular distribution of HO-1 in an *in vivo* model of chronic liver injury and to investigate the role of HO-1 in the liver cell types involved in the inflammatory and fibrotic response to chronic liver injury, the hepatic stellate cells and macrophages.

Materials and Methods

Animals and surgery

Specified pathogen-free male Wistar rats were purchased from Charles River Laboratories Inc. (Wilmington, MA, USA). Rats were housed under standard laboratory conditions with free access to standard laboratory chow and water. Rats were subjected to bile duct ligation as described previously.(23,24) Bile duct ligation was confirmed by determination of plasma bilirubin levels. Sham-operated animals were used as control. Animals were sacrificed 4 days and 1, 2 and 4 weeks after bile duct ligation. Experiments were approved by the local Committee for Care and Use of laboratory animals.

Hepatic stellate cell isolation and cell culture

Hepatic stellate cells (HSCs) were isolated from male Wistar rats (500-600 g) by pronase (Merck, Amsterdam, the Netherlands) and collagenase-P (Roche, Almere, The Netherlands) perfusion of the liver, followed by Nycodenz (Axis-Shield POC, Oslo,

Norway) gradient (13% w/v) centrifugation as described previously.(25) Purity of the cell population was estimated at 90% after isolation, and 100% after 1 day of culturing as studied with auto-fluorescence. Cells were then cultured in Iscove's Modified Dulbecco's Medium with Glutamax (Invitrogen, Breda, The Netherlands) supplemented with 20% heat-inactivated fetal calf serum (Invitrogen), 1 mmol/L sodium-pyruvate (Invitrogen), 1x MEM non-essential amino acids (Invitrogen), 50 μg/mL gentamycin (Invitrogen), 100 U/mL penicillin (Lonza, Vervier, Belgium), 10 μg/mL streptomycin (Lonza), 250 ng/mL fungizone (Lonza) in a humidified atmosphere containing 5% CO_2 at 37 °C. In order to study activated HSCs, the cells were seeded on plastic plates to allow culture-activation. Cells were passaged after 7 days and passage 1 cells were used for experiments.

To study macrophages we used Raw 264.7 cells, a mouse macrophage cell line as well as primary rat Kupffer cells. Raw 264.7 cells were cultured in Dulbecco's Modified Eagle Medium with Glutamax (Invitrogen, Breda, The Netherlands) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen), 50 μg/mL gentamycin (Invitrogen), 2mmol/L L-glutamine (Invitrogen) in a humidified atmosphere containing 5% CO_2 at 37 °C. Kupffer cells were isolated from male Wistar rats (150-250 gr), using collagenase liver perfusion. A Percoll density cushion (25-50%) was used to separate the non-parenchymal cell fraction as described before.(26) Kupffer cells were purified from the non-parenchymal cell fraction by selective adherence to tissue culture plates for 30 min in HBSS containing Mg^{+} and Ca^{2+} supplemented with 10% FCS. After 30 min the supernatant was removed, and contaminating cells were washed away and Kupffer cells were cultured in RPMI supplemented with 10% FCS. Purity of the cell population was confirmed using ED1 staining. After 4h of culture, cells were used for experiments. Experiments and infections were performed in serum free conditions.

Overexpression of heme oxygenase-1 in stellate cells and macrophages

To induce HO-1 expression in cells, two approaches were used. A recombinant adenovirus containing HO-1 cDNA (AdHO-1) was used to infect HSCs (MOI 1000) and Raw cells (MOI 1500). A LacZ/β-gal-containing adenovirus was used as a control virus. The Ad5HO-1 adenovirus was a kind gift of prof. Augustine Choi, University of Pittsburg, Pittsburg, USA and has been described and used before.(18,27,28)

To induce HO-1 expression in macrophages a high MOI is needed in order to saturate scavenger receptors of these cells. This high MOI could lead to side effects and compromise the functionality of the Kupffer cells. Therefore we used the pharmacological agent hemin to induce HO-1 expression in Kupffer cells using a concentration of 10 μmol/L.

Western Blot analysis

Western blot analysis of total cell lysates was performed by SDS-PAGE followed by semi dry-blotting to transfer the proteins to Hybond ECL nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). Protein expression of HO-1 was detected using an antibody against HO-1 (OSA-111, mouse monoclonal, Stressgen Biotechnologies Stressgen) at a dilution of 1:500. To confirm equal loading, we used a monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Calbiochem, dilution 1:10000) or β-actin (Sigma, dilution 1:5000). The blots were analyzed in a ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA). Protein band intensities were quantified by Image Lab (Bio-Rad).

RNA isolation and quantitative real time PCR

RNA was isolated using Tri-reagent (Sigma-Aldrich) according to the manufacturer's instructions. Reverse transcription was performed on 1 μg of total RNA using random nanomers (Sigma-Aldrich) in a final volume of 50 μL.

Real time detection was performed on the ABI PRISM 7700 (PE Applied Biosystems) using the Taqman protocol. This protocol includes an initiation of 10 min at 95°C, followed by 40 cycles (15 seconds at 95°C, and 1 minute at 60°C). mRNA levels of 18S were used as an internal control. Relative gene expressions were calculated using the ΔΔCt method. The primers (Invitrogen) and probes (Eurogentec) used, are described in table 1.

Immunofluorescence microscopy

We performed co-staining of paraffin-embedded liver tissue for HO-1, αSMA and ED-1 using a sequential fluorescence method. For HO-1/αSMA staining, we performed antigen retrieval using microwave irradiation in citrate buffer, pH 6.0. For HO-1/ED1 we used overnight incubation with Tris/HCl pH 9.0. The following primary antibodies were used: HO-1 (SPA-896, rabbit polyclonal, Stressgen Biotechnologies; dilution 1:100), αSMA (α-smooth muscle actin, a marker for stellate cell activation; mouse monoclonal antibody, Sigma Aldrich; dilution 1:1000) and ED-1 (a marker for macrophages; mouse-anti-rat monoclonal antibody CD68, MCA 341R, Serotec; dilution 1:750). As secondary antibodies we used Alexa fluorophores; goat anti-rabbit Ig (Alexa Fluor 555, Invitrogen, 1:500 diluted in PBS/1% BSA, against HO-1 expressing a red colour), goat anti-mouse Ig (Alexa Fluor 488, Invitrogen, 1:500 diluted in PBS/1% BSA, against α-SMA and ED-1, expressing a green colour). Finally, the slides were mounted in fluorescence mounting

medium S3023 (DAKO). The slides were analyzed using a confocal laser scanning microscope (Zeiss 410 inverted laser scan microscope).

Statistical analysis

Results are presented as the mean of a least 3 independent experiments ±SD. A student t-test or a one way ANOVA test with Bonferroni as post-test was used to determine the significance of differences between experimental groups. A P-value of less than 0,05 was considered statistically significant. Calculations were made using the software of GraphPad Prism 5.00.

Table 1 Sequences of primers and probes used for quantitative PCR analysis

Gene		Primers
18S rat/mouse	Sense	5′-CGG CTA CCA CAT CCA AGG A-3′
	Anti-sense	5′-CCA ATT ACA GGG CCT CGA AA-3′
	Probe	5′-CGC GCA AAT TAC CCA CTC CCG A -3′
αSMA rat	Sense	5′-GCC AGT CGC CAT CAG GAA C-3′
	Anti-sense	5′-CAC ACC AGA GCT GTG CTG TCT T-3′
	Probe	5′-CTT CAC ACA TAG CTG GAG CAG CTT CTC GA-3′
Collagen type 1 rat	Sense	5′-TGG TGA ACG TGG TGT ACA AGG T-3′
	Anti-sense	5′-CAG TAT CAC CCT TGG CAC CAT-3′
	Probe	5′-TCC TGC TGG TCC CCG AGG AAA CA-3′
COX2 mouse/rat	Sense	5′-TTG TTG AGT CAT TCA CCA GAC AGA T-3′
	Anti-sense	5′-GCC TTT GCC ACT GCT TGT ACA-3′
	Probe	5′-CCC CAG CAA CCC GGC CAG C-3′
HO-1 rat/mouse	Sense	5′-CAC AGG GTG ACA GAA GAG GCT AA-3′
	Anti-sense	5′-CTG GTC TTT GTG TTC CTC TGT CAG-3′
	Probe	5′-CAG CTC CTC AAA CAG CTC AAT GTT GAG C-3′
iNOS mouse	Sense	5′-CTA TCT CCA TTC TAC TAC TAC CAG ATC GA-3′
	Anti-sense	5′-CCT GGG CCT CAG CTT CTC AT-3′
	Probe	5′-CCC TGG AAG ACC CAC ATC TGG CAG-3′
iNOS rat	Sense	5′-GTG CTA ATG CGG AAG GTC ATG-3′
	Anti-sense	5′-CGA CTT TCC TGT CTC AGT AGC AAA-3′
	Probe	5′-CCC GCG TCA GAG CCA CAG TCC T-3′
MRC1 mouse	Sense	5′-GCT ATT GGA CGC GAG GCA AT-3′
	Anti-sense	5′-CGT CTG AAC TGA GAT GGC ACT TAG-3′
	Probe	5′-CCA CGC AGC GCT TGT GAT CTT CA-3′
MRC1 rat		Taqman assay on demand (Invitrogen)
TGFβ mouse	Sense	5′-GGG CTA CCA TGC CAA CTT CTG-3′
	Anti-sense	5′-GAG GGC AAG GAC CTT GCT GTA-3′
	Probe	5′-CCC TGC CCC TAT ATT TGG AGC CTG GAC-3′
TGFβ rat	Sense	5′-GGG CTA CCA TGC CAA CTT CTG-3′
	Anti-sense	5′-GAG GGC AAG GAC CTT GCT GTA-3′
	Probe	5′-CCT GCC CCT ACA TTT GGA GCC TGG A-3′
TNF-α mouse	Sense	5′-GTA GCC CAC GTC GTA GCA AAC-3′
	Anti-sense	5′-AGT TGG TTG TCT TTG AGA TCC ATG-3′
	Probe	5′-CGC TGG CTC AGC CAC TCC AGC-3′
TNFα rat	Sense	5′-GTA GCC CAC GTC GTA GCA AAC-3′
	Anti-sense	5′-AGT TGG TTG TCT TTG AGA TCC ATG-3′
	Probe	5′-CGCTGGCTCAGCCACTCCAGC-3′

Results

HO-1 is expressed by macrophages after chronic liver injury

Previously, we demonstrated that HO-1 mRNA expression is increased in total rat liver RNA after bile duct ligation(18). To determine which hepatic cell type(s) contributes to the increased HO-1 mRNA expression, we performed immunofluorescence for HO-1, αSMA and ED1 (marker of macrophages) on liver from rats that underwent BDL surgery from 4 days up to 4 weeks. As shown in **Figure 1A**, αSMA was mainly expressed around vessels and in the portal areas around proliferating bile ducts, representing staining in myofibroblasts. HO-1 expression was localized in the parenchyma. No colocalisation of HO-1 and αSMA was observed at any time point after bile duct ligation.

Since the staining pattern of HO-1 resembled that of inflammatory cells, we performed double staining of HO-1 and ED1. As shown in **Figure 1B**, there is co-localisation of HO-1 and ED1, indicating that the most likely source of HO-1 in the liver in this model are macrophages, including Kupffer cells, the liver-specific macrophages. This co-localisation is complete in early stages of BDL (after 4 days), but at later stages (after 4 weeks of BDL) an increasing number of HO-1 negative, ED1 positive cells is observed. This suggests that the HO-1 expression in macrophages is transient and that it might have a regulatory function in the inflammatory response.

Overexpression of HO-1 in macrophages causes a shift from M2 to M1 type macrophages

To further study a possible regulatory effect of HO-1 in macrophages, we investigated HO-1 expression in Raw 264.7 cells, a mouse macrophage cell line, and primary rat Kupffer cells. Both Raw 264.7 cells and Kupffer cells had a basal level of HO-1 expression. (data not shown)

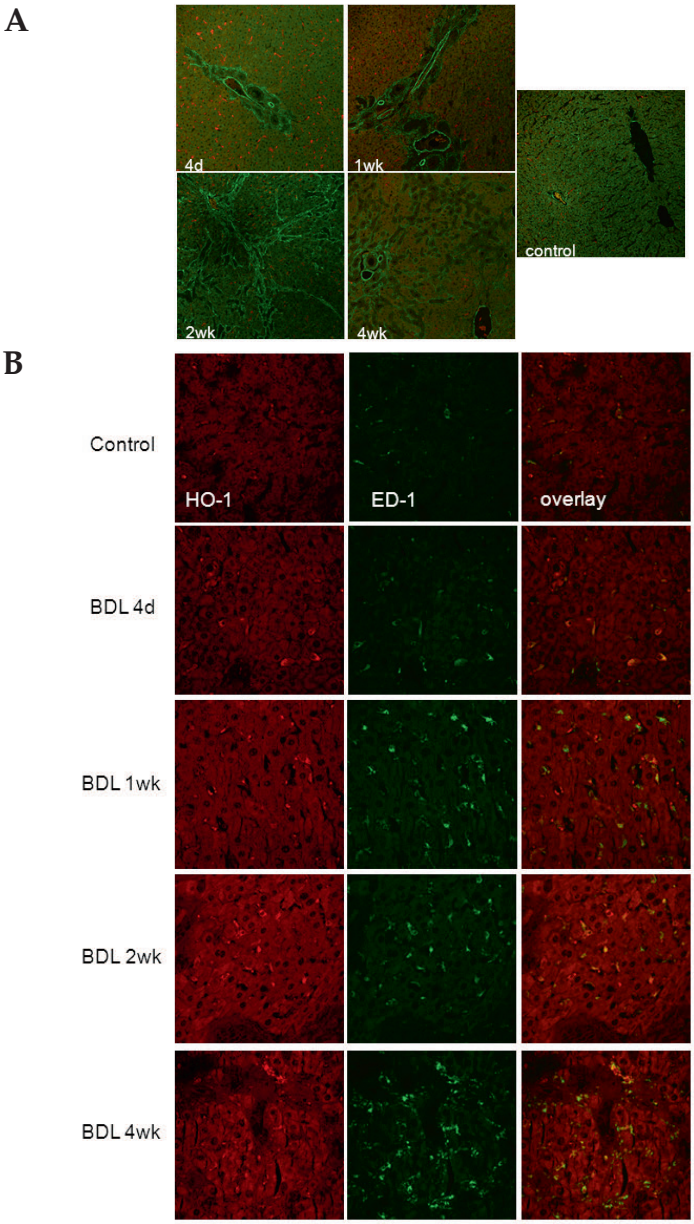
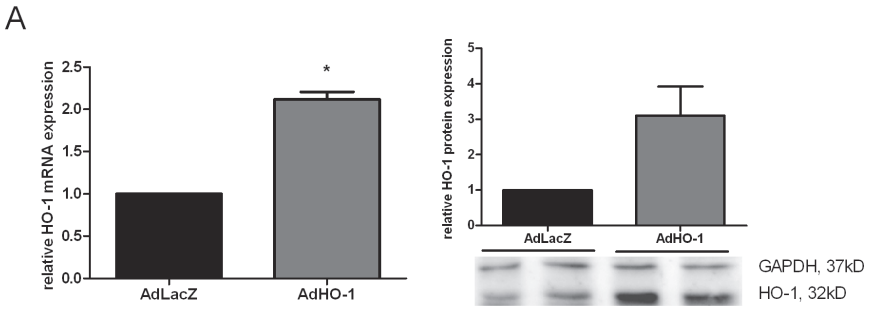


Figure 1 HO-1 expression is predominantly localised in macrophages
(A) Immunofluorescence on liver sections of rats subjected to bile duct ligation or sham operation. HO-1 (red staining) is predominantly expressed intraparenchymally, α-SMA (green staining) is expressed around vessels and in portal areas. No co-localisation of HO-1 and α-SMA was detected. Magnification of 630x.
(B) Immunofluorescence on liver sections of rats subjected to bile duct ligation, or sham-operated rats. ED-1 (green staining) is predominantly expressed intraparenchymally, and co-localises with HO-1 staining (red). After 4 weeks of BDL the co-localisation is not complete, there are ED-1 positive cells, which are HO-1 negative. Magnification 630x.

Overexpression of HO-1 in Raw cells was achieved using the adenoviral vector AdHO-1 **(Figure 2A)**. To induce HO-1 expression in primary Kupffer cells we used hemin, since functionality of Kupffer cells may be compromised by viral infection **(Figure 2B)**.

Since HO-1 is known to be involved in inflammatory reactions and may have an immunomodulatory role, we investigated whether HO-1 overexpression modulated markers that are associated with the phenotype of macrophages. Upon HO-1 induction in Raw cells **(Figure 2C)** as well as in primary rat Kupffer cells **(Figure 2D)**, transforming growth factor-β (TGFβ) and mannose receptor 1 (MRC1) mRNA levels (markers for M2 type macrophages) decreased, whereas tumor necrosis factor-α (TNFα), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) mRNA levels (markers associated with the M1 type macrophage) increased. These differences are statistically significant ($p<0,05$).



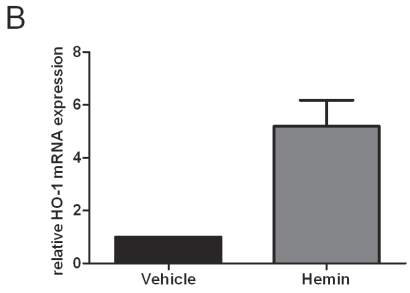
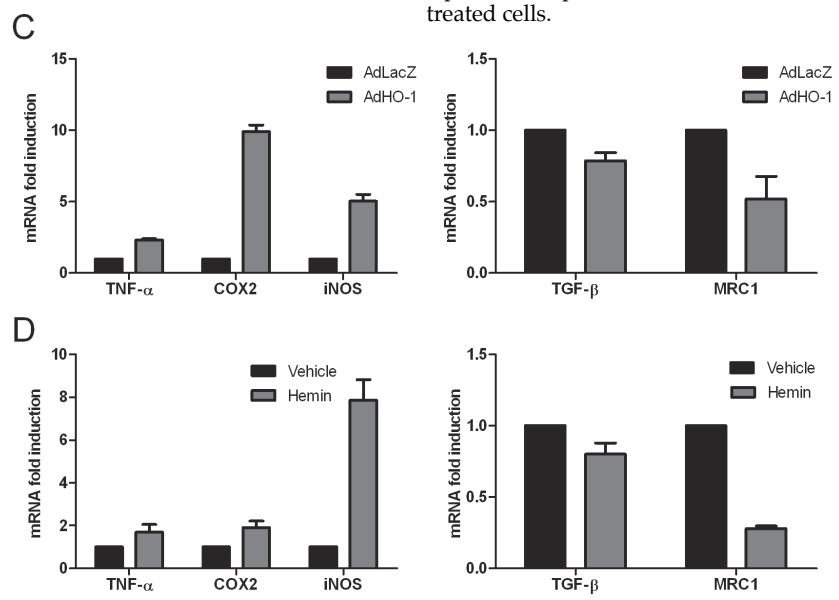


Figure 2 Induction of HO-1 in macrophages causes a shift from M2 to M1 type macrophage phenotype
A) AdHO-1 (MOI 1000, 72h) infection leads to significantly increased levels AdHO-1 (MOI 1000, 72h) infection leads to significantly increased levels of HO-1 mRNA and protein in Raw cells compared to AdLacZ-infected cells.
B) Hemin (10μM) induces HO-1 mRNA expression in primary rat Kupffer cells compared to vehicle treated cells.
C) TNF-α, iNOS and COX-2 mRNA levels are increased upon induction of HO-1 in RAW cells whereas TGF-β and MRC1 mRNA levels are reduced compared to AdLacZ infected Raw cells.
D) TNF-α, COX2 and iNOS mRNA expression is increased, whereas TGF-β expression is reduced upon induction of HO-1 expression in primary Kupffer cells.
* p<0,05 compared to AdLacZ-transfected, or vehicle treated cells.



Expression of α-smooth muscle actin and collagen type 1 is not modified by HO-1 overexpression in primary hepatic stellate cells

Since expression of HO-1 in myofibroblasts has been reported before (20) and since we did not observe co-staining of HO-1 and αSMA in bile duct-ligated liver, we examined HO-1 expression in stellate cells in more detail. The basal mRNA (data not shown) and protein expression (**Figure 3A)** of HO-1 in activated stellate cells was very low to absent. We next examined the effect of forced expression on stellate cell markers using an adenovirus overexpressing HO-1 (AdHO-1) induced HO-1 mRNA as well as protein levels. **(Figure 3A)**

Overexpression of HO-1 in activated primary rat hepatic stellate cells did not significantly modulate the expression of the fibrogenic markers αSMA or collagen type 1 **(Figure 3B)**.

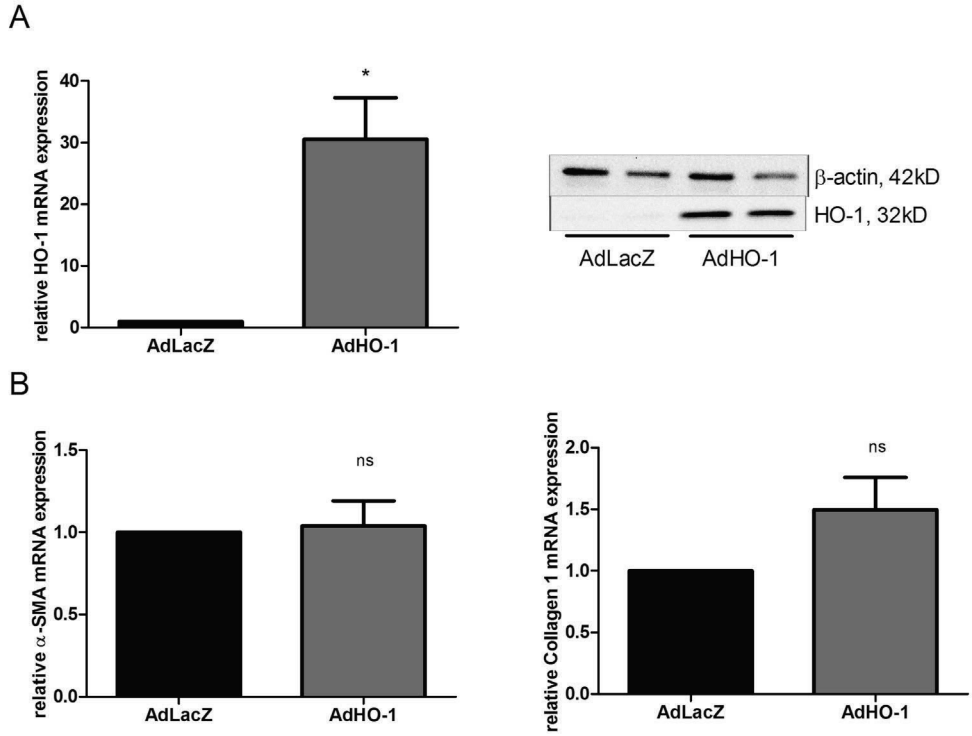


Figure 3. HO-1 overexpression in primary rat hepatic stellate cells does not influence activation
(A) AdHO-1 (MOI 1000, 72h) leads to significantly increased levels of HO-1 mRNA and protein in primary rat hepatic stellate cells compared to cells infected with the control virus AdLacZ.
(B) Overexpression of HO-1 did not change activation markers α-SMA and collagen type 1 compared to AdLacZ transfection. * p<0,05 compared to AdLacZ-transfected cells.

Discussion

In this study, we investigated the expression and role of heme oxygenase-1 in the *in vivo* model of cholestatic chronic liver disease: bile duct ligation. We also investigated the expression and role of HO-1 *in vitro*, focusing on the liver cell types most intimately involved in the inflammatory and fibrotic response: macrophages and hepatic stellate cells.

In the *in vivo* model of chronic liver disease we studied, bile duct ligation, the only cells expressing HO-1 are macrophages. This is most likely due to an early inflammatory response accompanying the acute cholestatic injury. Others have also shown an induction of HO-1 expression in Kupffer cells after BDL compared to sham operated rats.(29,30)

In addition, two studies reported HO-1 expression only in Kupffer cells in normal rat and human liver.(31-33) Our results agree with these observations; however after chronic liver injury we observed an increase in the number of cells expressing both HO-1 and ED-1, as well as an increase in the intensity of expression. The increase in the number of cells expressing both these markers could be explained by an increased influx of macrophages (for example from the bone-marrow) or by an increase of a specific subpopulation of macrophages. In addition, it is of interest to note that the initial complete co-localisation of HO-1 and ED-1 after liver injury seems to disappear at later stages of the injury. Possibly, HO-1 has a regulatory function in the macrophages, priming them for their initial actions after injury, but is not necessary anymore after prolonged injury.

It is known that macrophages have dual roles in modulating progression and resolution of liver fibrosis (i.e. by production of TGF-β and platelet-derived growth factor (PDGF), matrix metalloproteinases (MMPs) and tissue inhibitor metalloproteinases (TIMPs), phagocytosis of debris etc.), through their ability to change their phenotype. (34,35)

Classically, macrophages act pro-inflammatory, producing cytokines like tumor necrosis factor α (TNF-α), interleukin 6 (IL-6) and interleukin-1β (IL-1β), a phenotype known as M1 phenotype. On the other hand, macrophages can change to the M2 phenotype, following alternative activation, producing cytokines which are more involved in wound remodelling (and hence fibrogenesis), such as interleukin 10 (IL-10) or transforming growth factor β (TGF-β).

In this study, we show that an induction of HO-1 in macrophages caused a shift of the macrophage phenotype from M2 to M1 phenotype, thus from a profibrotic to

a pro-inflammatory phenotype. This shift would lead to an increased inflammatory response, which may appear as an undesired reaction. However, we hypothesize that an (initial) inflammatory microenvironment is favourable over a profibrotic response, because it will accelerate the resolution and/or clearance of the causative agent of the inflammatory reaction, thereby limiting long-term activation of stellate cells and fibrosis. This is supported by recent reports, that showed an increased phagocytosis and bacterial clearance (both associated with the M1-phenotype) upon carbon monoxide-induced HO-1 expression.(36-38) Previously, it was reported that HO-1 is able to modulate the phenotype of blood and alveolar macrophages to a M2 phenotype.(39-41). This is contradictory to our findings of liver macrophage modulation by HO-1 induction to the M1 phenotype, however, this may be due to differences in organ microenvironments or differences between macrophage populations in different organs.

Contrary to other studies we did not find any effect of HO-1 expression on the phenotype of hepatic stellate cells. The constitutive expression of HO-1 in hepatic stellate cells is very low to absent. Induction of HO-1 expression does not seem to have an effect on fibrogenic markers of stellate cells. Furthermore, upon liver damage *in vivo* HO-1 is not induced in stellate cells or other myofibroblasts. Others have demonstrated HO-1 expression in αSMA-positive hepatic myofibroblasts in human cirrhotic liver samples. (20) However, although these cells may resemble HSCs, they are not identical to activated HSCs and therefore a comparison is difficult.

In our study, overexpression of HO-1 in activated primary hepatic rat stellate cells did not significantly alter the expression of the activation markers αSMA and collagen type 1. This is contradictory to previous studies, claiming that an antifibrogenic effect was established upon induction of HO-1 in hepatic stellate cells using different strategies (15-d-PGJ2 (20), and 2',4',6'-tris(methoxymethoxy) chalcone (TMMC) (42)). These seemingly contradictory results can be explained by the non-specific HO-1 inducers used by others described above. For example HO-1 induction using the PPAR-γ agonist 15-d-PGJ2, could lead to antifibrogenic effects mediated by other PPAR-gamma targets.

In summary, our results demonstrate that HO-1 is predominantly expressed by macrophages in chronic liver disease *in vivo*. Forced overexpression of HO-1 in macrophages *in vitro* caused a phenotypic shift in macrophages, establishing an anti-fibrotic microenvironment. Indirectly, this possibly attenuates the activation of hepatic stellate cells, and as a consequence halts liver fibrogenesis.

Acknowledgements

Microscopic fluorescence imaging was performed at the UMCG Microscopy & Imaging Center (UMIC), which is supported by the Netherlands Organisation for Health Research and Development (ZonMW grant 40-00506-98-9021).

References

(1) Clark JM. The epidemiology of nonalcoholic fatty liver disease in adults. J Clin Gastroenterol 2006 Mar;40 Suppl 1:S5-10.

(2) Marchesini G, Bugianesi E, Forlani G, Cerrelli F, Lenzi M, Manini R, et al. Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. Hepatology 2003 Apr;37(4):917-923.

(3) Lee KS, Lee SJ, Park HJ, Chung JP, Han KH, Chon CY, et al. Oxidative stress effect on the activation of hepatic stellate cells. Yonsei Med J 2001 Feb;42(1):1-8.

(4) Lee KS, Buck M, Houglum K, Chojkier M. Activation of hepatic stellate cells by TGF alpha and collagen type I is mediated by oxidative stress through c-myb expression. J Clin Invest 1995 Nov;96(5):2461-2468.

(5) Svegliati Baroni G, D'Ambrosio L, Ferretti G, Casini A, Di Sario A, Salzano R, et al. Fibrogenic effect of oxidative stress on rat hepatic stellate cells. Hepatology 1998 Mar;27(3):720-726.

(6) Nieto N, Friedman SL, Cederbaum AI. Stimulation and proliferation of primary rat hepatic stellate cells by cytochrome P450 2E1-derived reactive oxygen species. Hepatology 2002 Jan;35(1):62-73.

(7) Zamara E, Novo E, Marra F, Gentilini A, Romanelli RG, Caligiuri A, et al. 4-Hydroxynonenal as a selective pro-fibrogenic stimulus for activated human hepatic stellate cells. J Hepatol 2004 Jan;40(1):60-68.

(8) Galli A, Svegliati-Baroni G, Ceni E, Milani S, Ridolfi F, Salzano R, et al. Oxidative stress stimulates proliferation and invasiveness of hepatic stellate cells via a MMP2-mediated mechanism. Hepatology 2005 May;41(5):1074-1084.

(9) Guimaraes EL, Franceschi MF, Grivicich I, Dal-Pizzol F, Moreira JC, Guaragna RM, et al. Relationship between oxidative stress levels and activation state on a hepatic stellate cell line. Liver Int 2006 May;26(4):477-485.

(10) Sass G, Soares MC, Yamashita K, Seyfried S, Zimmermann WH, Eschenhagen T, et al. Heme oxygenase-1 and its reaction product, carbon monoxide, prevent inflammation-related apoptotic liver damage in mice. Hepatology 2003 Oct;38(4):909-918.

(11) Zeng B, Lin G, Ren X, Zhang Y, Chen H. Over-expression of HO-1 on mesenchymal stem cells promotes angiogenesis and improves myocardial function in infarcted myocardium. J Biomed Sci 2010 Oct 7;17:80.

(12) Correa-Costa M, Semedo P, Monteiro AP, Silva RC, Pereira RL, Goncalves GM, et al. Induction of heme oxygenase-1 can halt and even reverse renal tubule-interstitial fibrosis. PLoS One 2010 Dec 13;5(12):e14298.

(13) Morse D. The role of heme oxygenase-1 in pulmonary fibrosis. Am J Respir Cell Mol Biol 2003 Sep;29(3 Suppl):S82-6.

(14) Jozkowicz A, Was H, Dulak J. Heme oxygenase-1 in tumors: is it a false friend? Antioxid Redox Signal 2007 Dec;9(12):2099-2117.

(15) Lai IR, Chang KJ, Chen CF, Tsai HW. Transient limb ischemia induces remote preconditioning in liver among rats: the protective role of heme oxygenase-1. Transplantation 2006 05/15;81(0041-1337; 9):1311-1317.

(16) Jaeschke H. Molecular mechanisms of hepatic ischemia-reperfusion injury and preconditioning. Am J Physiol Gastrointest Liver Physiol 2003 Jan;284(1):G15-26.

(17) Redaelli CA, Tian YH, Schaffner T, Ledermann M, Baer HU, Dufour JF. Extended preservation of rat liver graft by induction of heme oxygenase-1. Hepatology 2002 May;35(5):1082-1092.

(18) Conde de la Rosa L, Vrenken TE, Hannivoort RA, Buist-Homan M, Havinga R, Slebos DJ, et al. Carbon monoxide blocks oxidative stress-induced hepatocyte apoptosis via inhibition of the p54 JNK isoform. Free Radic Biol Med 2008 Apr 1;44(7):1323-1333.

(19) Li L, Julien B, Grenard P, Teixeira-Clerc F, Mallat A, Lotersztajn S. Molecular mechanisms regulating the antifibrogenic protein heme-oxygenase-1 in human hepatic myofibroblasts. J Hepatol 2004 Sep;41(3):407-413.

(20) Li L, Grenard P, Nhieu JT, Julien B, Mallat A, Habib A, et al. Heme oxygenase-1 is an antifibrogenic protein in human hepatic myofibroblasts. Gastroenterology 2003 Aug;125(2):460-469.

(21) Willis D, Moore AR, Frederick R, Willoughby DA. Heme oxygenase: a novel target for the modulation of the inflammatory response. Nat Med 1996 Jan;2(1):87-90.

(22) Poss KD, Tonegawa S. Reduced stress defense in heme oxygenase 1-deficient cells. Proc Natl Acad Sci U S A 1997 Sep 30;94(20):10925-10930.

(23) Schoemaker MH, Gommans WM, Conde de la Rosa L, Homan M, Klok P, Trautwein C, et al. Resistance of rat hepatocytes against bile acid-induced apoptosis in cholestatic liver injury is due to nuclear factor-kappa B activation. J Hepatol 2003 Aug;39(2):153-161.

(24) Kountouras J, Billing BH, Scheuer PJ. Prolonged bile duct obstruction: a new experimental model for cirrhosis in the rat. Br J Exp Pathol 1984 Jun;65(3):305-311.

(25) Moshage H, Casini A, Lieber CS. Acetaldehyde selectively stimulates collagen production in cultured rat liver fat-storing cells but not in hepatocytes. Hepatology 1990 Sep;12(3 Pt 1):511-518.

(26) Teufelhofer O, Parzefall W, Kainzbauer E, Ferk F, Freiler C, Knasmuller S, et al. Superoxide generation from Kupffer cells contributes to hepatocarcinogenesis: studies on NADPH oxidase knockout mice. Carcinogenesis 2005 Feb;26(2):319-329.

(27) Fallaux FJ, Kranenburg O, Cramer SJ, Houweling A, Van OH, Hoeben RC, et al. Characterization of 911: a new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. Hum Gene Ther 1996 01/20;7(1043-0342; 2):215-222.

(28) McGrory WJ, Bautista DS, Graham FL. A simple technique for the rescue of early region I mutations into infectious human adenovirus type 5. Virology 1988 Apr;163(2):614-617.

(29) Wei CL, Lee KH, Khoo HE, Hon WM. Expression of haem oxygenase in cirrhotic rat liver. J Pathol 2003 Mar;199(3):324-334.

(30) Flores O, Criado M, Sanchez-Rodriguez A, Hidalgo F, Collia F, Lopez-Novoa JM, et al. Relationships between NOS2 and HO-1 in liver of rats with chronic bile duct ligation. Hepatol Res 2005 May;32(1):58-65.

(31) Bauer I, Rensing H, Florax A, Ulrich C, Pistorius G, Redl H, et al. Expression pattern and regulation of heme oxygenase-1/heat shock protein 32 in human liver cells. Shock 2003 Aug;20(2):116-122.

(32) Immenschuh S, Baumgart-Vogt E, Tan M, Iwahara S, Ramadori G, Fahimi HD. Differential cellular and subcellular localization of heme-binding protein 23/peroxiredoxin I and heme oxygenase-1 in rat liver. J Histochem Cytochem 2003 Dec;51(12):1621-1631.

(33) Geuken E, Buis CI, Visser DS, Blokzijl H, Moshage H, Nemes B, et al. Expression of heme oxygenase-1 in human livers before transplantation correlates with graft injury and function after transplantation. Am J Transplant 2005 Aug;5(8):1875-1885.

(34) Sica A, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. J Clin Invest 2012 Mar 1;122(3):787-795.

(35) Wynn TA, Barron L. Macrophages: master regulators of inflammation and fibrosis. Semin Liver Dis 2010 Aug;30(3):245-257.

(36) Chiang N, Shinohara M, Dalli J, Mirakaj V, Kibi M, Choi AM, et al. Inhaled carbon monoxide accelerates resolution of inflammation via unique proresolving mediator-heme oxygenase-1 circuits. J Immunol 2013 Jun 15;190(12):6378-6388.

(37) Onyiah JC, Sheikh SZ, Maharshak N, Steinbach EC, Russo SM, Kobayashi T, et al. Carbon monoxide and heme oxygenase-1 prevent intestinal inflammation in mice by promoting bacterial clearance. Gastroenterology 2013 Apr;144(4):789-798.

(38) Lee S, Lee SJ, Coronata AA, Fredenburgh L, Chung SW, Perrella M, et al. Carbon Monoxide Confers Protection in Sepsis by Enhancing Beclin 1-Dependent Autophagy and Phagocytosis. Antioxid Redox Signal 2013 Aug 25.

(39) Hualin C, Wenli X, Dapeng L, Xijing L, Xiuhua P, Qingfeng P. The anti-inflammatory mechanism of heme oxygenase-1 induced by hemin in primary rat alveolar macrophages. Inflammation 2012 Jun;35(3):1087-1093.

(40) Weis N, Weigert A, von Knethen A, Brune B. Heme oxygenase-1 contributes to an alternative macrophage activation profile induced by apoptotic cell supernatants. Mol Biol Cell 2009 Mar;20(5):1280-1288.

(41) Ma JL, Yang PY, Rui YC, Lu L, Kang H, Zhang J. Hemin modulates cytokine expressions in macrophage-derived foam cells via heme oxygenase-1 induction. J Pharmacol Sci 2007 Mar;103(3):261-266.

(42) Lee SH, Seo GS, Kim HS, Woo SW, Ko G, Sohn DH. 2',4',6'-Tris(methoxymethoxy) chalcone attenuates hepatic stellate cell proliferation by a heme oxygenase-dependent pathway. Biochem Pharmacol 2006 Nov 15;72(10):1322-1333.

Chapter 3

Glutathione and antioxidant enzymes serve complementary roles in protecting activated hepatic stellate cells against hydrogen peroxide-induced cell death

Marjolein H. Tiebosch[1], Sandra Dunning[1], Atta ur Rehman[1], Rebekka A. Hannivoort[1], Floris W. Haijer[1], Jannes Woudenberg[1], Fiona A.J. van den Heuvel[1], Manon Buist-Homan[1,2], Klaas Nico Faber[1,2], Han Moshage[1,2]

M.H.T., S.D. and A. u R. contributed equally to this work
K.N.F. and H.M. contributed equally to this work

[1]Department of Gastroenterology and Hepatology and [2]Department of Laboratory Medicine, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

Biochim Biophys Acta. 2013 Jul 16;1832(12):2027-2034

Abstract

Background: In chronic liver disease, hepatic stellate cells (HSCs) are activated, highly proliferative and produce excessive amounts of extracellular matrix, leading to liver fibrosis. Elevated levels of toxic reactive oxygen species (ROS) produced during chronic liver injury have been implicated in this activation process. Therefore, activated hepatic stellate cells need to harbor highly effective anti-oxidants to protect against the toxic effects of ROS.

Aim: To investigate the protective mechanisms of activated HSCs against ROS-induced toxicity.

Methods: Culture-activated rat HSCs were exposed to hydrogen peroxide. Necrosis and apoptosis were determined by Sytox Green or acridine orange staining, respectively. The hydrogen peroxide detoxifying enzymes catalase and glutathione-peroxidase (GPx) were inhibited using 3-amino-1,2,4-triazole (3AT) and mercaptosuccinic acid (MS), respectively. The anti-oxidant glutathione was depleted by L-buthionine-sulfoximine (BSO) and repleted with the GSH-analogue GSH-monoethylester (GSH-MEE).

Results: Upon activation, HSCs increase their cellular glutathione content and GPx expression, while MnSOD (both at mRNA and protein level) and catalase (at the protein level, but not at the mRNA level) decreased. Hydrogen peroxide did not induce cell death in activated HSCs. Glutathione depletion increased the sensitivity of HSCs to hydrogen peroxide, resulting in 35% and 75% necrotic cells at 0.2 and 1 mmol/L hydrogen peroxide, respectively. The sensitizing effect was abolished by GSH-MEE. Inhibition of catalase or GPx significantly increased hydrogen peroxide-induced apoptosis, which was not reversed by GSH-MEE.

Conclusion: Activated HSCs have increased ROS-detoxifying capacity compared to quiescent HSCs. Glutathione levels increase during HSC activation and protect against ROS-induced necrosis, whereas hydrogen peroxide-detoxifying enzymes protect against apoptotic cell death.

Introduction

Oxidative stress is defined as the imbalance between pro-oxidants and anti-oxidants. (1,2) Under normal conditions, reactive oxygen species are detoxified by various enzymatic and non-enzymatic antioxidants. When pro-oxidants exceed the antioxidant capacity of the cell, oxidative stress is the result.(1,2) Prolonged oxidative stress in the liver is associated with liver fibrosis and cirrhosis.(3-7) Liver fibrosis is characterized by the loss of hepatocytes and the activation of hepatic stellate cells (HSCs).(3-5) During the activation process quiescent HSCs transform into proliferating myofibroblast-like cells. Unlike quiescent HSCs, these activated cells lack retinoid-storing capacity, produce excessive amounts of connective tissue and proliferate.(4,5)

Although generation of reactive oxygen species has been implicated in the activation of stellate cells and liver fibrosis (6-16), little is known about the role of the different antioxidant systems in activated HSCs. Several enzymes are able to generate hydrogen peroxide, e.g. NADPH-oxidases and xanthine oxidase.(1,2) In addition, hydrogen peroxide is generated in the detoxification of superoxide anions by superoxide dismutases like the cytosolic CuZn-SOD (SOD1) and the mitochondrial Mn-SOD (SOD2). (1,2) Pathophysiological conditions often lead to increased hydrogen peroxide levels produced by inflammatory cells, e.g. neutrophils.(1) Hydrogen peroxide is detoxified by catalase that resides in peroxisomes or by cytosolic glutathione peroxidase. Glutathione peroxidase converts reduced glutathione (GSH) into oxidized glutathione (GSSG).(1,2) To control the hydrogen peroxide level within the cell, the cell has to balance the activity of catalase and glutathione peroxidases relative to SODs. The aim of this study was to investigate the role of antioxidant systems in the resistance of stellate cells to hydrogen peroxide-induced toxicity.

Material and Methods

Animals and surgery

Specified pathogen-free male Wistar rats were purchased from Harlan (Zeist, the Netherlands). They were housed under standard laboratory conditions and had free access to standard laboratory chow and water. Each experiment was performed following the guidelines of the local committee for care and use of laboratory animals.

Hepatic stellate cell isolation and culture

Hepatic stellate cells (HSCs) were isolated from male Wistar rats (500-600 g) by pronase (Merck, Amsterdam, the Netherlands) and collagenase-P (Roche, Almere, the Netherlands) perfusion of the liver, followed by Nycodenz (Axis-Shield POC, Oslo, Norway) gradient (12% w/v) centrifugation as described previously.(17) Cells were then cultured in Iscove's Modified Dulbecco's Medium with Glutamax (Invitrogen, Breda, the Netherlands) supplemented with 20% heat-inactivated fetal calf serum (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 1x MEM non-essential amino acids (Invitrogen), 50 μg/mL gentamycin (Invitrogen), 100 U/mL penicillin (Lonza, Vervier, Belgium), 10 μg/mL streptomycin (Lonza), 250 ng/mL fungizone (Lonza) and 250 U/mL Nystatin (Sanofi-Synthelabo, Maassluis, the Netherlands) in a humidified atmosphere containing 5% CO_2 at 37°C. For studying activation of hepatic stellate cells, cells were seeded, grown to confluency and harvested at the indicated time points. Primary HSC cultures were passaged via trypsinization, and then cultured in Iscove's medium with supplements as described above, except Nystatin. Prior to experiments, HSCs were serum-starved for 24 hours, unless indicated otherwise.

Experimental design

HSCs were culture-activated on tissue culture plastic for at least 7 days. The activated rat HSCs were exposed to oxidative stress induced by 0.2 or 1 mM hydrogen peroxide (Merck) or 20 or 50 μM menadione (Sigma-Aldrich, the Netherlands). The glutathione depleting compound L-buthionine-sulfoximine (BSO, Sigma-Aldrich, the Netherland) was used at 200 μM. The cell permeable glutathione donor GSH-monoethylester (GSH-MEE, Calbiochem, VWR, The Netherlands) was used at 5 mM. The glutathione peroxidase inhibitor mercaptosuccinic acid (MS, Sigma-Aldrich) was used at 10 mM and the catalase inhibitor 3-amino-1,2,4-triazole (3AT, Sigma-Aldrich) was used at 20 mM. The caspase-3 inhibitor (Z-DEVD-FMK004R & D Systems, Abingdon UK) was used at 0.05 μM. Inhibitors were added 30 minutes prior to exposure to hydrogen peroxide, with the exception of BSO, which was added 17-20 hours prior to exposure to hydrogen peroxide.

Glutathione assay

Glutathione and glutathione disulfide content were determined using a spectrophotometry-based assay as described previously.(18,19) HSCs were harvested in a lysis buffer composed of 25 mM HEPES, 5 mM $MgCl_2$, 5 mM EDTA, 2 mM PMSF, 10 μg/μL pepstatin and 10 μg/μL leupeptin and then lysed by 3 cycles of snap-freezing in liquid nitrogen and thawing. Values were corrected for protein concentration, determined by the BioRad DC protein assay (Veenendaal, the Netherlands) according to the manufacturer's instructions.

RNA isolation

RNA was isolated using Tri-reagent (Sigma-Aldrich) according to the manufacturer's instructions. Reverse transcription was performed on total RNA using random nonamers (Sigma-Aldrich) in a final volume of 50 μl. Reverse transcription was performed in three steps: 10 minutes at 25°C, 1 hour at 37°C and 5 minutes at 95°C.

Quantitative Real-Time PCR

Real time detection was performed on the ABI PRISM 7700 (PE Applied Biosystems, the Netherlands) initialized by 10 min at 95 °C, followed by 40 cycles (15 seconds at 95°C, and 1 minute at 60°C). Each sample was analyzed in duplicate. mRNA levels of 18S were used as an internal control. Reaction mixture contained qPCR mastermix plus-dTTP (Eurogentec, Maastricht, the Netherlands) supplemented with 900 nM sense and anti-sense primer and 200 nM labeled probe. The primers (Invitrogen) and probe (Eurogentec) used are listed in **Table 1**. Relative gene expressions were calculated using the ΔΔCt method.

Western blot analysis

Western blot analysis was performed as described previously.(19) Equal amounts of protein were loaded on SDS-PAGE gels. Proteins were transferred using semi-dry electrophoretic transfer. Specific proteins were detected using primary antibodies: mouse anti-GAPDH (1:10,000, Calbiochem, VWR, the Netherlands CB1001), mouse anti-α-smooth muscle actin (1:2,000, Sigma Aldrich), rabbit polyclonal anti-Mn-superoxide dismutase (1:1,000, Stressgen, Enzo life Sciences, Antwerpen, Belgium, SOD-111), polyclonal rabbit anti-catalase (1:2,000, Calbiochem 219010), polyclonal rabbit anti-Pex-14 (1:2000, generous gift of Dr. M. Fransen, Leuven, Belgium), and polyclonal rabbit anti-β-

actin (1:2000, Sigma-Aldrich A2066). Protein bands were detected using a Chemidoc XRS system (Bio-Rad).

Table 1 Sequences of primers and probes used for quantitative PCR analysis

Gene		Primers
18S	Sense	5′-CGG CTA CCA CAT CCA AGG A-3′
	Anti-sense	5′-CCA ATT ACA GGG CCT CGA AA-3′
	Probe	5′-CGC GCA AAT TAC CCA CTC CCG A -3′
α-SMA	Sense	5′-GCC AGT CGC CAT CAG GAA C-3′
	Anti-sense	5′-CAC ACC AGA GCT GTG CTG TCT T-3′
	Probe	5′-CTT CAC ACA TAG CTG GAG CAG CTT CTC GA-3′
Catalase	Sense	5′-GGA TTA TGG CCT CCG AGA TCT-3′
	Anti-sense	5′-ACC TTG GTC AGG TCA AAT GGA T-3′
	Probe	5′-ATG CCA TCG CCA GTG GCA ATT ACC-3′
Collagen 1a1	Sense	5′-TGG TGA ACG TGG TGT ACA AGG T-3′
	Anti-sense	5′-CAG TAT CAC CCT TGG CAC CAT-3′
	Probe	5′-TCC TGC TGG TCC CCG AGG AAA CA-3′
CuZnSOD	Sense	5′-CAG GAC CTC ATT TTA ATC CTC ACT C-3′
	Anti-sense	5′-GTC TCC AAC ATG CCT CTC TTC A-3′
	Probe	5′-CCG CTG GAC CGC CAT GTT TCT T-3′
GCL (γ-GCS)	Sense	5′-GCC CAA TTG TTA TGG CTT TGA GT-3′
	Anti-sense	5′-CCT CCC GTG TTC TAT CAT CTA CAG A-3′
	Probe	5′-ACT CCC CAG CGA CAA TCA ATG TCT GAC AC-3′
GPx1	Sense	5′-GGACATCAGGAGAATGGCAAGA-3′
	Anti-sense	5′-CGCACTTCTCAAACAATGTAAAGTTG-3′
	Probe	5′-TTCCCTCAAGTATGTCCGACCCGGTG-3′
HO-1	Sense	5′-CAC AGG GTG ACA GAA GAG GCT AA-3′
	Anti-sense	5′-CTG GTC TTT GTG TTC CTC TGT CAG-3′
	Probe	5′-CAG CTC CTC AAA CAG CTC AAT GTT GAG C-3′
MnSOD	Sense	5′-CAC CGA GGA GAA GTA CCA CGA-3′
	Anti-sense	5′-GAA CTT CAG TGC AGG CTG AAG A-3′
	Probe	5′-CCT GAG TTG TAA CAT CTC CCT TGG CCA G-3′
TGF-β	Sense	5′-GGG CTA CCA TGC CAA CTT CTG-3′
	Anti-sense	5′-GAG GGC AAG GAC CTT GCT GTA-3′
	Probe	5′-CCT GCC CCT ACA TTT GGA GCC TGG A-3′

Apoptosis and necrosis determination by Acridine orange and Sytox green/Hoechst 33342 nuclear staining.

Cells were seeded in 12-well plates and treated as indicated. Apoptosis was determined by assessment of nuclear condensation using Acridine orange staining (Sigma-Aldrich) at 2.5 μg/mL. After 6 hours, the percentage of apoptotic cells was determined by dividing the number of condensed nuclei by the total number of nuclei

per field, amplified with 100. Percentages are the mean of two randomly chosen fields per condition (magnification 200 x; 15 nuclei per field).

To determine necrosis, HSCs were incubated with Sytox green nucleic acid staining (Invitrogen, Breda ,The Netherland) at 125 nM in combination with Hoechst 33342 (Roche, Almere, the Netherlands) at 5 μg/mL. Sytox green penetrates cells with leaky plasma membranes, a hallmark of necrotic cells, but does not cross the plasma membranes of viable or apoptotic cells and has been validated before (19). Hoechst 33342 crosses the plasma membrane of all cells. After 3 hours, the percentage of necrotic cells was determined by dividing the number of Sytox green positive nuclei by the number of Hoechst 33342 positive nuclei of the same field, amplified by 100. Two randomly-chosen fields were used to determine the average per condition (magnification 200x; 15 nuclei per field). Cells were monitored using an Olympus CKX41 microscope at 450-490 nm.

Immunofluorescence microscopy

Cells were fixed with 4% paraformaldehyde, labeled and analyzed as described previously (20). Rabbit polyclonal antibodies against catalase (dilution 1:200; Calbiochem) or MnSOD (dilution 1:100, Stressgen) were use as primary antibodies, followed by secondary antibodies labeled with Alexa Fluor 568 or Alexa Fluor 488 (Invitrogen), respectively. Images were captured with a Leica TCS SP2-AOBS confocal laser scanning microscope (Leica, Heidelberg, Germany).

Statistical analysis

Statistical analyses of data were performed using SPSS 14. Data are presented as mean ± standard deviation, unless otherwise indicated. Statistical differences between groups were calculated using the non-parametric Kruskal-Wallis test, followed by a Mann-Whitney-U-test. P-values below 0.05 were considered significant.

Results

Glutathione peroxidase 1 expression is induced during activation of hepatic stellate cells

Marked changes in gene expression occur when quiescent HSCs transform into activated HSCs. Therefore, we first investigated the expression of various genes involved in the detoxification of reactive oxygen species during the activation process. As expected, alpha-smooth muscle actin (α-SMA), a marker for HSC activation, was strongly induced upon activation **(Figure 1A)**. mRNA levels of catalase were significantly induced after two days of culture, but decreased upon complete activation of stellate cells **(Figure 1B)**. Manganese superoxide dismutase (Mn-SOD) mRNA levels progressively declined upon activation **(Figure 1C)**, while the copper-zinc superoxide dismutase (CuZn-SOD) expression remained unaltered **(Figure 1D)**. Interestingly, mRNA expression of glutathione peroxidase 1 (GPx1) increased during activation of HSCs **(Figure 1E)**, while glutamate-cysteine ligase (GCL), the rate-limiting enzyme in glutathione synthesis, was not altered upon HSC activation **(Figure 1F)**. HO-1 expression in HSCs was very low and although a trend towards reduced heme oxygenase-1 (HO-1) expression was observed, this did not reach statistical significance **(Figure 1G)**.

The mRNA data were confirmed by analyzing the levels of the corresponding proteins by Western blotting **(Figure 1H)**. Alpha-smooth muscle actin, β-actin and Gapdh protein expression all increased upon HSC activation. In contrast, Mn-SOD protein levels sharply decreased during activation of HSC. Similarly, also catalase protein levels dropped immediately after day 1, which was not observed for the catalase mRNA levels. Despite the low protein levels of catalase and MnSOD as detected by Western blotting, both proteins remained detectable using immunocytochemistry and revealed a typical peroxisomal and mitochondrial location, respectively **(Figure 2)**. Given the large and unexpected difference in β-actin and Gapdh levels in quiescent versus activated HSC, we searched for alternative proteins to use as loading control for Western blot analysis. Pex14p, a protein involved in translocating proteins into peroxisomes, showed a stable signal relative to 18S mRNA level and total protein loaded for Western blot analysis **(Figure 1H, bottom panel)**.

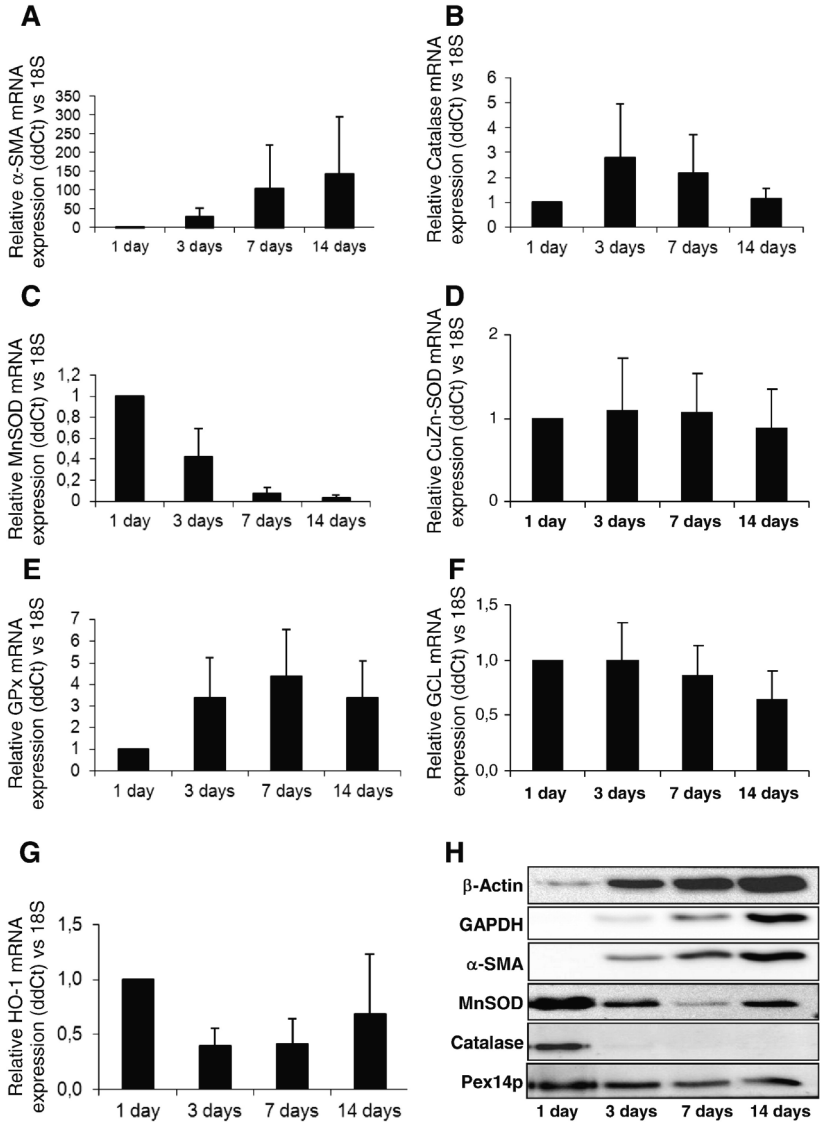


Figure 1. Expression of ROS-detoxifying enzymes during hepatic stellate cell activation
Primary rat HSC were culture-activated for 1, 3, 7 and 14 days and mRNA (**A-G**) and protein (**H**) levels of activation markers and anti-oxidant enzymes were quantified by RT-Q-PCR and western blotting, respectively. α-SMA mRNA (**A**) and protein (**H**) levels progressively increased during HSC activation. Catalase mRNA levels (**B**) transiently increased, while the corresponding protein rapidly declined in total cell extracts after day 1 (**H**). Expression of Mn-SOD progressively decreased during HSC activation, both at mRNA (**C**) and protein (**H**) level, while CuZn-SOD levels did not change (**D**). The expression of GPx1 increased (**E**), while the expression of GCL did not change (**F**, **H**). The expression of HO-1 was low and not significantly changed during HSC activation (**G**). Pex14p was used as loading control for Western blot analysis, since β-actin and GAPDH protein levels strongly increased upon HSC activation (**H**), The Western blots are representative of four independent experiments. mRNA data was corrected for 18S and is presented as means ± SD. *Significant difference compared to quiescent HSCs at day 1, $p<0.05$.

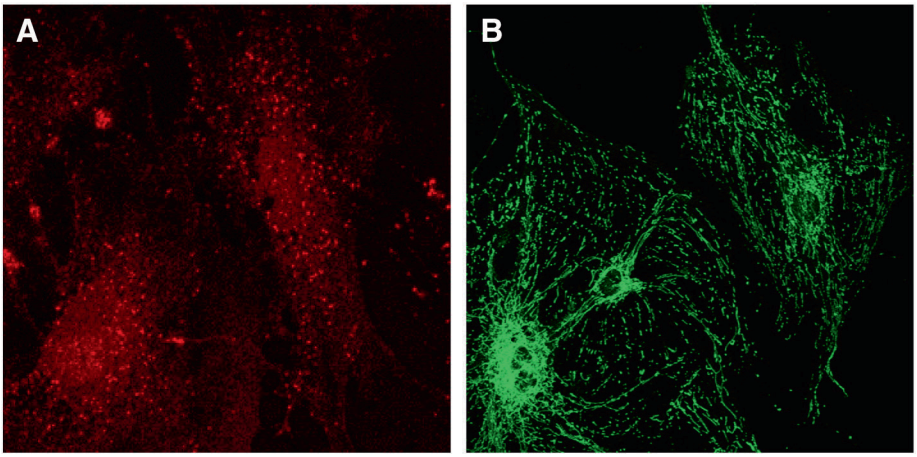


Figure 2. Catalase and Mn-SOD protein is still detectable in activated HSC.
Fully activated HSC were analyzed for the presence and subcellular location of catalase (**A**) and MnSOD (**B**) using immunofluorescence microscopy. A punctate staining typical for a peroxisomal location was observed for catalase (red stain), while MnSOD staining (green) was restricted to mitochondria. Magnification 630x.

Activated hepatic stellate cells have a higher glutathione content than quiescent hepatic stellate cells

Total glutathione levels were determined in quiescent (1 day after isolation) HSCs and in fully activated (>7 days after isolation) HSCs. The total cellular glutathione content was increased 5.6-times upon activation of HSC from 0.18 μmol/μg protein in quiescent HSC to 1.0 μmol/μg protein in activated HSC **(Figure 3)**, despite unchanged GCL mRNA levels, the rate-limiting enzyme in the synthesis of glutathione **(Figure 1)**. Subsequent analysis revealed that 88% of the total glutathione content was in the reduced (GSH) form (mean of 3 different isolates of HSCs).

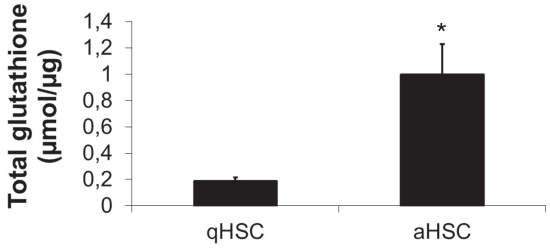


Figure 3. Total glutathione content is increased upon hepatic stellate cell activation
Total glutathione levels were quantified in quiescent (qHSC, 1 day in culture) and fully activated (aHSC, > 7 days in culture) HSC. Activated HSCs have approximately 5.6-fold higher glutathione levels than quiescent HSCs. Results are shown as mean ± S.E.M of four independent experiments, * significantly different from quiescent stellate cells, $p<0.05$.

Glutathione depletion moderately increases oxidative stress in activated hepatic stellate cells

Pre-incubation of activated, serum-starved HSCs with BSO reduced total glutathione levels by 87% (Figure 4A). No increase was observed in either necrotic or apoptotic cell death (data not shown) as well as no gross changes in cellular morphology was observed in BSO-treated HSC. Likewise, BSO treatment also reduced glutathione content by 88% in activated HSCs cultured in medium containing 20% FCS (data not shown), without visible morphological changes.

To investigate whether glutathione depletion leads to increased oxidative stress in HSCs, we determined the mRNA level of the oxidative stress-responsive gene heme-oxygenase-1 (HO-1). Depletion of glutathione increased HO-1 mRNA levels 2.3-fold **(Figure 4B)**, which is only minor when compared to the induction of HO-1 by menadione or hydrogen peroxide (5-50 fold) (6,19). In addition, mRNA expression of GCL, the rate-limiting enzyme in glutathione synthesis, was increased only 1.6-fold **(Figure 4B)**. In contrast, glutathione depletion had no effect on the expression of the hydrogen peroxide-detoxifying enzymes GPx1 and catalase and the superoxide dismutases Mn-SOD, and CuZn-SOD **(Figure 4B,C)**. These data indicate that glutathione depletion alone only minimally induces markers of oxidative stress. Furthermore, glutathione depletion did not change the expression of the known markers of stellate cell activation α-smooth muscle actin (α-SMA), collagen type 1, and TGF-β **(Figure 4D)**. Finally, glutathione depletion or glutathione supplementation, using GSH-MEE did not alter stellate cell proliferation (data not shown). Next, we analyzed whether glutathione depletion sensitizes HSCs for oxidative stress.

Depletion of glutathione increases sensitivity to hydrogen peroxide-induced necrosis

After depletion of glutathione with BSO, 35% and 75% of the cells became necrotic after a 3 hour exposure to 0.2 or 1 mM hydrogen peroxide, respectively **(Figure 5)**. This indicates that glutathione depletion greatly enhanced sensitivity to hydrogen peroxide-induced necrosis. Restoration of glutathione content, using GSH-MEE, almost completely reversed hydrogen peroxide-induced necrosis in BSO-treated HSCs **(Figure 5)**.

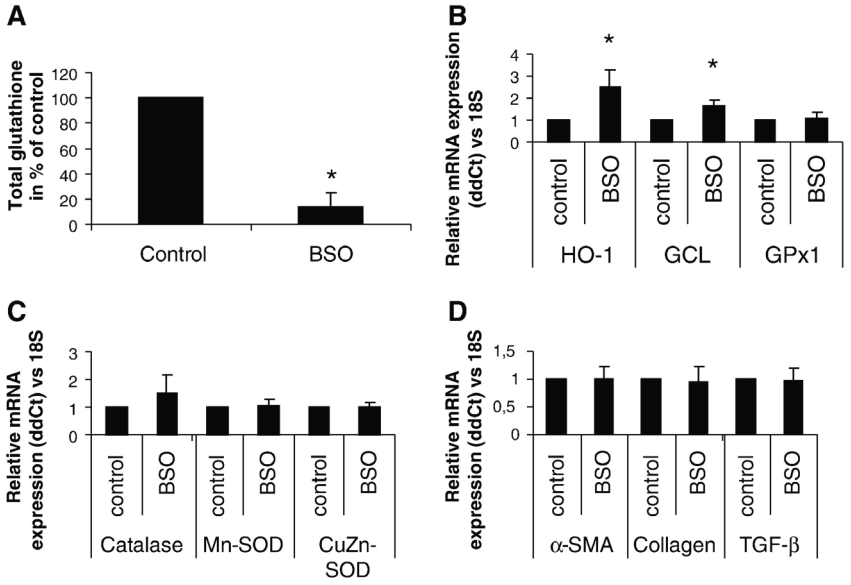


Figure 4. Glutathione depletion moderately increases oxidative stress in activated hepatic stellate cells

Fully-activated HSC were treated with BSO for 20 h and analyzed for total glutathione content (**A**) and mRNA levels of anti-oxidant enzymes (**B,C**) and activation markers (**D**). Pre-treating HSCs with BSO depleted cellular glutathione content by 87% in serum-starved HSCs (**A**). BSO treatment moderately induced HO-1 and GCL and had no effect on GPx1 (**B**) nor on the expression of the anti-oxidant enzymes catalase, Mn-SOD, and CuZn-SOD (**C**) or the expression of the HSC activation markers, α-SMA, collagen type 1 and TGF-β (**D**). Results are shown as mean ± st. dev. of at least four independent experiments, * significantly different from controls; $p<0.05$.

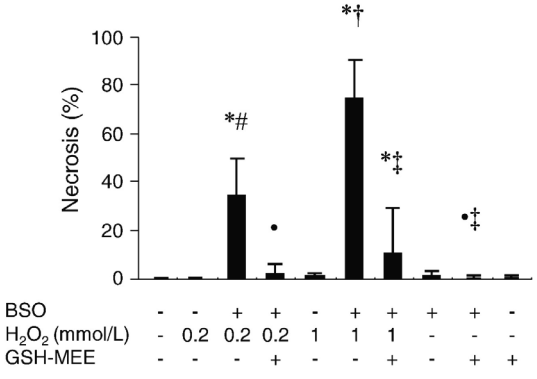


Figure 5. Glutathione depletion increases sensitivity of hepatic stellate cells to hydrogen peroxide-induced necrosis

Fully-activated HSCs were exposed for 3 h to H_2O_2 in the absence or presence of BSO and/or GSH-MEE and analyzed for necrotic cell death by Sytox green nuclear staining. BSO treatment followed by exposure to 0.2 mM or 1 mM hydrogen peroxide (H_2O_2) induced necrosis in 35% and 75% of the cells, respectively. Co-treatment with GSH-MEE almost completely prevented the induction of HSC necrosis by BSO+H_2O_2. Results are shown as mean ± st. dev. of four independent experiments. *Significant difference compared to control, $p<0.05$; # Significant difference compared to 0.2 mM hydrogen peroxide, $p<0.05$; •Significant difference compared to 0.2 mM hydrogen peroxide + BSO, $p<0.05$; †Significant difference compared to 1 mM hydrogen peroxide, $p<0.05$; ‡Significant difference compared to 1 mM hydrogen peroxide + BSO, $p<0.05$.

Glutathione peroxidase and catalase protect against oxidative stress-induced apoptosis

The importance of the antioxidant enzyme GPx in the protection of activated HSCs against oxidative stress was investigated using the GPx inhibitor mercaptosuccinic acid (MS). Inhibition of GPx using mercaptosuccinic acid increased apoptosis in the absence of hydrogen peroxide and in the presence of 0.2 mM and 1 mM hydrogen peroxide **(Figure 6A)**. No effects on necrosis were observed under these conditions (data not shown).

The importance of the antioxidant enzyme catalase in the protection of activated HSCs against oxidative stress was investigated using the catalase inhibitor 3-amino-1,2,4-triazole (3-AT). This inhibitor significantly induced apoptotic cell death (18%) in HSCs after 6 hours, even in the absence of exogenous hydrogen peroxide **(Figure 6A)**. Cells treated with 0.2 or 1 mM hydrogen peroxide in the presence of the inhibitor catalase showed even higher levels of apoptosis, 22% an 44% respectively **(Figure 6A)**. No effects on necrosis were detected at this time point.

To investigate the importance of the hydrogen peroxide detoxifying enzymes catalase and GPx in the protection against superoxide anions that are converted into hydrogen peroxide by superoxide dismutases, we exposed activated stellate cells to the superoxide anion generator menadione. As described previously, menadione at 20 μM induced predominantly apoptotic cell death, which was reduced by the glutathione donor GSH-MEE.(19) Inhibition of catalase in the presence of menadione had no effect on apoptotic cell death **(Figure 6C)**, but slightly increased necrotic cell death (data not shown), whereas inhibition of GPx did neither modulate apoptotic (**Figure 6C**) nor necrotic cell death (data not shown). Prior reduction of cellular glutathione levels by BSO did not aggravate apoptotic cell death induced by 20 μM menadione. Menadione at 50μM resulted in detachment of cells and massive necrotic cell death. (data not shown)

Combined inhibition of glutathione peroxidase and catalase elevates apoptosis of HSCs

Inhibiting both GPx and catalase by cotreatment with MS and 3AT resulted in 22% apoptotic cells after 6 hours even in the absence of exogenous hydrogen peroxide **(Figure 6A)**. Exposing these cells to hydrogen peroxide induced apoptosis even further: to 49% in co-treatment with 0.2 mM hydrogen peroxide and to 62 % in co-treatment with 1 mM hydrogen peroxide **(Figure 6A)**. Increasing the glutathione content using the glutathione donor GSH-MEE did not change the cell viability under these conditions **(Figure 6B)**. Inhibition of caspase-3 partially decreased apoptosis of HSCs **(Figure 6B)**. Necrotic cell death was not significantly enhanced in the described conditions (data not shown).

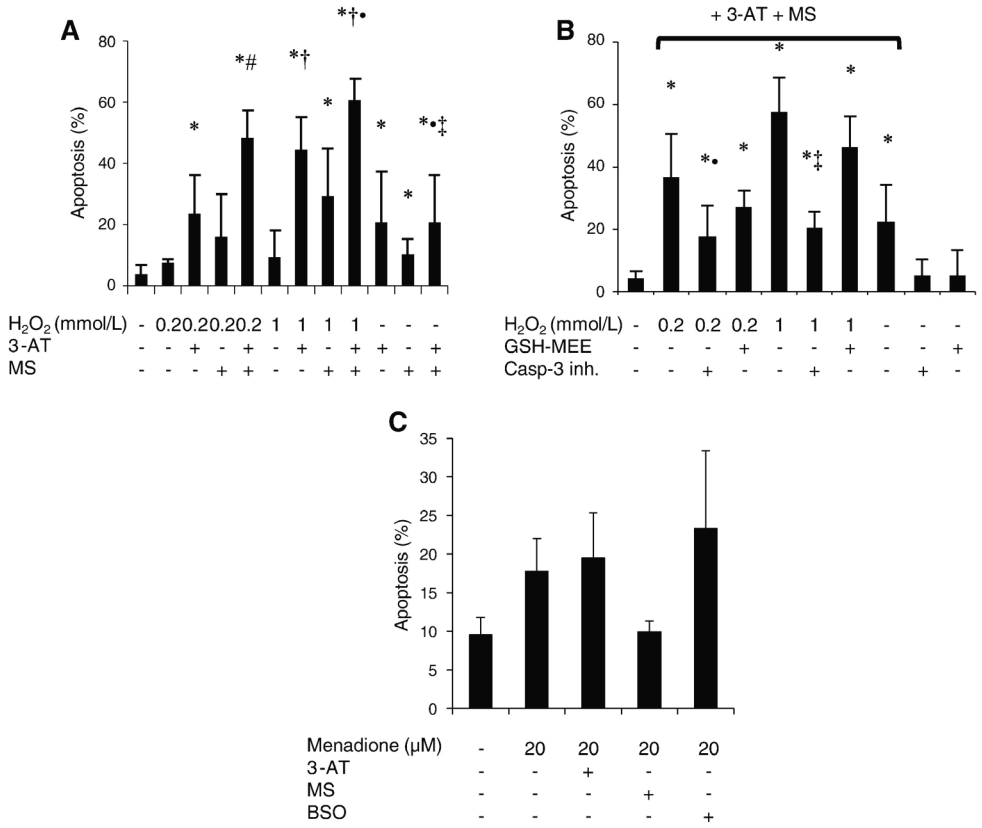


Figure 6. Inhibition of glutathione peroxidase and/or catalase induces apoptosis in activated hepatic stellate cells

Fully-activated HSCs were exposed for 6 h to H_2O_2 in the presence or absence of the catalase inhibitor 3-AT and/or GPx inhibitor MS **(A)**, as well as in the presence or absence of GSH-MEE or the caspase-3 inhibitor Z-DEVD-FMK **(B)** and analyzed for apoptotic cell death by acridine orange staining. Values are given as percentage apoptotic nuclei. 3-AT and MS treatment alone significantly increased the number of apoptotic HSC, which was further enhanced by cotreatment with H_2O_2 **(A)**. Apoptosis induced by treatment with H_2O_2 in the presence of inhibitors of GPx and catalase is inhibited by a blocker of caspase-3 activity, but not by supplementing glutathione through GSH-MEE **(B)**. Results are shown as mean ± stdev of at least four independent experiments. *Significant difference compared to control, $p<0.05$; ^Significant difference compared to MS, $p<0.05$; §Significant difference compared to 3AT, $p<0.05$; † Significant difference compared to 1 mM hydrogen peroxide, $p<0.05$; °Significant difference compared to MS + 3AT, $p<0.05$; #Significant difference compared to 0.2 mM hydrogen peroxide, $p<0.05$; •Significant difference compared to 0.2 mM hydrogen peroxide + 3-AT + MS, $p<0.05$; ‡Significant difference from 0.2 mmol/L hydrogen peroxide + 3-AT + MS, $p<0.05$; ⁿSignificant difference from 1 mmol/L hydrogen peroxide + 3-AT + MS, $p<0.05$.

(C) Fully-activated HSCs were exposed for 9 h to 20μM menadione in the presence or absence of the catalase inhibitor 3-AT, the GPx inhibitor MS, as well as the glutathione depleting agent BSO analyzed for apoptotic cell death by acridine orange staining. Values are given as percentage apoptotic nuclei. Menadione induced apoptotic cell death at 20 μM. 3-AT and MS did not significantly modulate apoptotic cell death by menadione. Moreover, depletion of glutathione using BSO did not aggravate menadione-induced apoptotic cell death. Results are shown as mean ± st. dev. of at least four independent experiments.

Discussion

Chronic liver injury is almost invariably accompanied by increased oxidative stress, activation of stellate cells and fibrogenesis.(3-7) Activated HSCs must be well protected against oxidative stress, since they survive and proliferate in the chronically injured liver. The oxidative stress in chronically injured liver is composed of several reactive oxygen species, including hydrogen peroxide and superoxide anions. In this study, we have investigated the resistance of hepatic stellate cells to hydrogen peroxide-induced injury. We demonstrate that this resistance is to a large extent due to a high intracellular glutathione content and increased expression of glutathione peroxidase in activated stellate cells. Although an increased glutathione content in activated stellate cells has been reported before (21), the implications have never been investigated in the context of oxidative stress-induced cell death.

Maher *et al*. showed, in addition to increased glutathione levels upon activation, an increase in the activity and mRNA level of glutamate-cysteine ligase (GCL), the rate-limiting enzyme in glutathione synthesis.(21) We did not observe an induction of GCL mRNA upon stellate cell activation in our experiments. The increased cellular glutathione content is most likely due to a higher activity of GCL, especially since GCL activity is known to be regulated by the glutathione content.(22) It is unlikely that the increased glutathione content is due to reduced activity of the GSSG export pump multidrug resistance protein 1 (Mrp1), since we have previously shown that the expression of this transporter is increased in activated stellate cells and contributes significantly to the survival of activated stellate cells.(23)

Upon HSC activation, the expression of the hydrogen peroxide detoxifying enzyme glutathione peroxidase 1 (GPx1) is increased. This increase may be an adaptive response to oxidative stress. Indeed, mice over-expressing GPx1 are better protected against oxidative stress and they survive concentrations of the oxidant paraquat that are lethal in wild type mice and even more harmful in GPx1 knockout mice.(24,25) Mice that overexpress GPx1 are also more resistant to oxidative stress due to myocardial ischemia-reperfusion injury(26) It should be noted that glutathione is essential for the activity of GPx, since GPx converts reduced glutathione into oxidized glutathione. This might explain the coordinated increase in cellular glutathione content and GPx expression during the activation process of hepatic stellate cells. Therefore, our data suggest that activated hepatic stellate cells may be more resistant against oxidative stress than

quiescent stellate cells. Interestingly, upon activation the mRNA expression level of the mitochondrial superoxide anion converting enzyme Mn-SOD is reduced, both at the mRNA and at the protein level. This finding is partially at variance with previous reports that revealed an initial increase in MnSOD mRNA expression, followed by a steady decrease of MnSOD mRNA expression.(27) Disruption of the Mn-SOD gene, a known tumor suppressor gene, is lethal in mice, which is a direct result of mitochondrial dysfunction, leading to metabolic acidosis, ketosis and accumulation of lipids in the liver and skeletal muscle.(28,29) Characterization of the heterozygous Mn-SOD knockout mice revealed no compensatory increase in other ROS-detoxifying enzymes, like glutathione peroxidase, CuZn-SOD or catalase.(30) Since Mn-SOD is restricted to the mitochondria, changes in its activity may not affect other components of the antioxidant defense system in other cellular compartment like the cytoplasm.(30) At present it is not known how the activated stellate cells detoxify the reactive oxygen species generated in mitochondria. One possibility is that the residual MnSOD protein content, as determined by Western-blotting and immunofluorescence, is sufficient to detoxify ROS generated in mitochondria. The reduction in Mn-SOD mRNA expression during activation could be due to the strong reduction of the transcription factor peroxisome proliferator-activated receptor-γ (PPAR-γ) that occurs during stellate cell activation. (data not shown and (31)) MnSOD expression is controlled by PPAR-γ: in PPAR-γ knockout mice, the expression of Mn-SOD is also reduced (32) and activation of PPAR-γ with the agonists rosiglitazone or rosuvastatin enhanced MnSOD activity and expression (33,34).

The catalase mRNA levels transiently increased during the activation process of HSC, which is largely in line which observations made by De Bleser et al. who analyzed catalase regulation during HSC activation by Northern blot analysis.(27) However, in contrast to the mRNA levels, the cellular catalase protein content sharply dropped 1 day after plating HSC. A typical peroxisomal staining of catalase remained detectable in fully activated HSC, indicating that some residual catalase protein was present in these cells. However, clearly, the catalase protein level was not an accurate reflection of the catalase mRNA content. At present it is unclear why the catalase protein disappears from activated stellate cells, but this may be a result of a rapid and selective degradation of this antioxidant protein. The low catalase levels did not sensitize activated HSCs to acute H_2O_2-induced necrosis, indicating the presence of an alternative H_2O_2 scavenging mechanism(s) in activated HSCs. However, blocking catalase activity by 3-AT made HSCs highly sensitive for apoptotic cell death, which was further enhanced by exposure to 0.2

or 1.0 mM H_2O_2. A similar effect was observed after blocking GPx activity, indicating that these 2 proteins play a key role in regulating apoptotic cell death in HSCs.

HSCs increase their glutathione levels upon activation. However, glutathione depletion has no direct effect on stellate cell morphology (35) and in this study, we show that glutathione depletion also has no effect on the expression of markers of stellate cell activation, such as collagen type I, α-SMA and TGF-β or anti-oxidant genes like catalase, Mn-SOD, CuZn-SOD, and GPx1. The glutathione levels per se do not seem to be a key mechanism in developing the activated phenotype.

Although increased mRNA levels of GCL and HO-1 were observed after glutathione depletion, these increases were modest, indicating that depletion of glutathione alone does not exert significant oxidative stress on stellate cells. Induction of GCL mRNA levels after glutathione depletion has also been shown in lung epithelial cells (36), endothelial cells (37), and in rat liver *in vivo* (38). Although HO-1 has been reported to inhibit HSC proliferation via p38 activation (39), we did not find an alteration in the proliferation rate of stellate cells after HO-1 induction due to glutathione depletion. Possibly, the induction of HO-1 by glutathione depletion in our study is too modest to have an effect on p38 phosphorylation and subsequent HSC proliferation. The induction of HO-1 by glutathione depletion was only 2.3-fold, whereas Li et al showed at least a 10-fold induction of HO-1 expression using 15-deoxy-delta-12,14-prostaglandin J2.(39,40)

Although glutathione depletion per sé had no effect on stellate cell viability, glutathione depletion increased the sensitivity of the cells to hydrogen peroxide-induced necrosis. Replenishing glutathione reduced necrotic cell death, without a shift towards apoptosis. Inhibition of the hydrogen peroxide-detoxifying activity by inhibitors of glutathione peroxidase or catalase induced HSC apoptosis, both in the absence and in the presence of exogenous hydrogen peroxide. Apoptotic cell death as a result of combined inhibition of glutathione peroxidase and catalase was shown to be independent of glutathione content, but partially dependent on caspase-3 activity. The superoxide anion donor menadione dose-dependently induces apoptotic cell death in activated HSCs.(19) Inhibition of hydrogen peroxide detoxifying enzymes did not significantly modulate menadione-induced cell death, indicating that increased superoxide generation did not lead to a massive, superoxide dismutase-mediated, increase in hydrogen peroxide.

The observed difference in mode of cell death, necrosis after glutathione depletion and apoptosis after inhibition of hydrogen peroxide detoxifying enzymes, may be explained by the cellular redox state. Glutathione is the most important regulator of the

cellular redox state.(41,42) Changes in the glutathione content will affect redox status and is known to influence activation of MAP-kinases, transcription factors and caspases. (41-43) In the presence of glutathione, caspases that require reduced cysteine-sulfhydryl groups in their catalytic site, can still be activated when the enzymes GPx and catalase are inhibited, because this inhibition is not likely to change the redox state of the cell. In contrast, in the absence of glutathione, e.g. after glutathione depletion, cells exposed to hydrogen peroxide cannot activate caspases and the apoptotic program and cell death is shifted towards necrosis. Such a shift from apoptotic to necrotic cell death has been reported before in hepatocytes exposed to superoxide anions.(44)

In summary, our study reveals important changes in the defense against oxidative stress of hepatic stellate cells during activation. These changes are characterized by increased cellular glutathione content and GPx1 mRNA expression. Furthermore, we demonstrate that both glutathione and the hydrogen peroxide-converting enzymes GPx and catalase are important in the resistance against hydrogen peroxide-induced cell death. Our data suggests that activated hepatic stellate cells *in vivo* may acquire increased resistance to necrotic cell death, while remaining sensitive to apoptosis, providing an explanation for their survival in the fibrotic liver and their apoptotic clearance during reversal of fibrosis.

Acknowledgements

Microscopic fluorescence imaging was performed at the UMCG Microscopy & Imaging Center (UMIC), which is supported by the Netherlands Organisation for Health Research and Development (ZonMW grant 40-00506-98-9021).

References

(1) VB Djordjevic. Free radicals in cell biology, Int.Rev.Cytol. 237 (2004) 57-89.

(2) M Inoue, EF Sato, M Nishikawa, AM Park, Y Kira, I Imada, K Utsumi. Mitochondrial generation of reactive oxygen species and its role in aerobic life, Curr.Med.Chem. 10 (2003) 2495-2505.

(3) D Wu, AI Cederbaum. Alcohol, oxidative stress, and free radical damage, Alcohol Res.Health. 27 (2003) 277-284.

(4) SL Friedman. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver, Physiol.Rev. 88 (2008) 125-172.

(5) AY Hui, SL Friedman. Molecular basis of hepatic fibrosis, Expert Rev.Mol.Med. 5 (2003) 1-23.

(6) H Tsukamoto, R Rippe, O Niemela, M Lin. Roles of oxidative stress in activation of Kupffer and Ito cells in liver fibrogenesis, J.Gastroenterol.Hepatol. 10 Suppl 1 (1995) S50-3.

(7) KS Lee, SJ Lee, HJ Park, JP Chung, KH Han, CY Chon, SI Lee, YM Moon. Oxidative stress effect on the activation of hepatic stellate cells, Yonsei Med.J. 42 (2001) 1-8.

(8) KS Lee, M Buck, K Houglum, M Chojkier. Activation of hepatic stellate cells by TGF alpha and collagen type I is mediated by oxidative stress through c-myb expression, J.Clin.Invest. 96 (1995) 2461-2468.

(9) G Svegliati Baroni, L D'Ambrosio, G Ferretti, A Casini, A Di Sario, R Salzano, F Ridolfi, S Saccomanno, AM Jezequel, A Benedetti. Fibrogenic effect of oxidative stress on rat hepatic stellate cells, Hepatology. 27 (1998) 720-726.

(10) N Nieto, SL Friedman, AI Cederbaum. Stimulation and proliferation of primary rat hepatic stellate cells by cytochrome P450 2E1-derived reactive oxygen species, Hepatology. 35 (2002) 62-73.

(11) E Zamara, E Novo, F Marra, A Gentilini, RG Romanelli, A Caligiuri, G Robino, E Tamagno, M Aragno, O Danni, R Autelli, S Colombatto, MU Dianzani, M Pinzani, M Parola. 4-Hydroxynonenal as a selective pro-fibrogenic stimulus for activated human hepatic stellate cells, J.Hepatol. 40 (2004) 60-68.

(12) A Galli, G Svegliati-Baroni, E Ceni, S Milani, F Ridolfi, R Salzano, M Tarocchi, C Grappone, G Pellegrini, A Benedetti, C Surrenti, A Casini. Oxidative stress stimulates proliferation and invasiveness of hepatic stellate cells via a MMP2-mediated mechanism, Hepatology. 41 (2005) 1074-1084.

(13) ELM Guimarães, MFS Franceschi, I Grivicich, F Dal-Pizzol, JCF Moreira, RM Guaragna, R Borojevic, R Margis, FC Guma. Relationship between oxidative stress levels and activation state on a hepatic stellate cell line, Liver International. 26 (2006) 477-485.

(14) E Novo, F Marra, E Zamara, L Valfre di Bonzo, A Caligiuri, S Cannito, C Antonaci, S Colombatto, M Pinzani, M Parola. Dose dependent and divergent effects of superoxide anion on cell death, proliferation, and migration of activated human hepatic stellate cells, Gut. 55 (2006) 90-97.

(15) KY Kim, I Choi, SS Kim. Progression of hepatic stellate cell activation is associated with the level of oxidative stress rather than cytokines during CCl4-induced fibrogenesis, Mol.Cells. 10 (2000) 289-300.

(16) P Greenwel, JA Dominguez-Rosales, G Mavi, AM Rivas-Estilla, M Rojkind. Hydrogen peroxide: a link between acetaldehyde-elicited alpha1(I) collagen gene up-regulation and oxidative stress in mouse hepatic stellate cells, Hepatology. 31 (2000) 109-116.

(17) H Moshage, A Casini, CS Lieber. Acetaldehyde selectively stimulates collagen production in cultured rat liver fat-storing cells but not in hepatocytes, Hepatology. 12 (1990) 511-518.

(18) OW Griffith. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine, Anal.Biochem. 106 (1980) 207-212.

(19) S Dunning, RA Hannivoort, JF de Boer, M Buist-Homan, KN Faber, H Moshage. Superoxide anions and hydrogen peroxide inhibit proliferation of activated rat stellate cells and induce different modes of cell death, Liver Int. 29 (2009) 922-932.

(20) A Pellicoro, FA van den Heuvel, M Geuken, H Moshage, PL Jansen, KN Faber. Human and rat bile acid-CoA:amino acid N-acyltransferase are liver-specific peroxisomal enzymes: implications for intracellular bile salt transport, Hepatology. 45 (2007) 340-348.

(21) JJ Maher, JM Saito, BA Neuschwander-Tetri. Glutathione regulation in rat hepatic stellate cells - Comparative studies in primary culture and in liver injury in vivo, Biochem.Pharmacol. 53 (1997) 637-641.

(22) GF Seelig, RP Simondsen, A Meister. Reversible dissociation of gamma-glutamylcysteine synthetase into two subunits, J.Biol.Chem. 259 (1984) 9345-9347.

(23) R.A. Hannivoort, S. Dunning, S. Vander Borght, B. Schroyen, J. Woudenberg, F. Oakley, M. Buist-Homan, F.A. van den Heuvel, M. Geuken, A. Geerts, T. Roskams, K.N. Faber & H. Moshage, Multidrug resistance-associated proteins are crucial for the viability of activated rat hepatic stellate cells, Hepatology 48 (2008) 624-634.

(24) H Jaeschke, YS Ho, MA Fisher, JA Lawson, A Farhood. Glutathione peroxidase-deficient mice are more susceptible to neutrophil-mediated hepatic parenchymal cell injury during endotoxemia: importance of an intracellular oxidant stress, Hepatology. 29 (1999) 443-450.

(25) XG Lei. Glutathione peroxidase-1 gene knockout on body antioxidant defense in mice, Biofactors. 14 (2001) 93-99.

(26) T Yoshida, M Watanabe, DT Engelman, RM Engelman, JA Schley, N Maulik, YS Ho, TD Oberley, DK Das. Transgenic mice overexpressing glutathione peroxidase are resistant to myocardial ischemia reperfusion injury, J.Mol.Cell.Cardiol. 28 (1996) 1759-1767.

(27) PJ De Bleser, GX Xu, K Rombouts, V Rogiers, A Geerts. Glutathione levels discriminate between oxidative stress and transforming growth factor-beta signaling in activated rat hepatic stellate cells, J.Biol.Chem. 274 (1999) 33881-33887.

(28) Y Li, TT Huang, EJ Carlson, S Melov, PC Ursell, JL Olson, LJ Noble, MP Yoshimura, C Berger, PH Chan, DL Wallace, CJ Epstein. Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase, Nat.Genet. 11 (1995) 376-381.

(29) RM Lebovitz, H Zhang, H Vogel, J Cartwright Jr, L Dionne, N Lu, S Huang, MM Matzuk. Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice, Proc.Natl. Acad.Sci.U.S.A. 93 (1996) 9782-9787.

(30) H Van Remmen, C Salvador, H Yang, TT Huang, CJ Epstein, A Richardson. Characterization of the antioxidant status of the heterozygous manganese superoxide dismutase knockout mouse, Arch.Biochem. Biophys. 363 (1999) 91-97.

(31) H She, S Xiong, S Hazra, H Tsukamoto. Adipogenic transcriptional regulation of hepatic stellate cells, J.Biol.Chem. 280 (2005) 4959-4967.

(32) G Ding, M Fu, Q Qin, W Lewis, HW Kim, T Fukai, M Bacanamwo, YE Chen, MD Schneider, DJ Mangelsdorf, RM Evans, Q Yang. Cardiac peroxisome proliferator-activated receptor gamma is essential in protecting cardiomyocytes from oxidative damage, Cardiovasc.Res. 76 (2007) 269-279.

(33) X Yu, XG Shao, H Sun, YN Li, J Yang, YC Deng, YG Huang. Activation of cerebral peroxisome proliferator-activated receptors gamma exerts neuroprotection by inhibiting oxidative stress following pilocarpine-induced status epilepticus, Brain Res. 1200 (2008) 146-158.

(34) W Verreth, D De Keyzer, PC Davey, B Geeraert, A Mertens, MC Herregods, G Smith, F Desjardins, JL Balligrand, P Holvoet. Rosuvastatin restores superoxide dismutase expression and inhibits accumulation of oxidized LDL in the aortic arch of obese dyslipidemic mice, Br.J.Pharmacol. 151 (2007) 347-355.

(35) JJ Maher, BA Neuschwander-Tetri. Manipulation of glutathione stores in rat hepatic stellate cells does not alter collagen synthesis, Hepatology. 26 (1997) 618-623.

(36) L Tian, MM Shi, HJ Forman. Increased Transcription of the Regulatory Subunit of γ-Glutamylcysteine Synthetase in Rat Lung Epithelial L2 Cells Exposed to Oxidative Stress or Glutathione Depletion, Arch. Biochem.Biophys. 342 (1997) 126-133.

(37) Y Urata, H Yamamoto, S Goto, H Tsushima, S Akazawa, S Yamashita, S Nagataki, T Kondo. Long exposure to high glucose concentration impairs the responsive expression of gamma-glutamylcysteine synthetase by interleukin-1beta and tumor necrosis factor-alpha in mouse endothelial cells, J.Biol.Chem. 271 (1996) 15146-15152.

(38) N Kiyosawa, K Ito, K Sakuma, N Niino, M Kanbori, T Yamoto, S Manabe, N Matsunuma. Evaluation of glutathione deficiency in rat livers by microarray analysis, Biochem.Pharmacol. 68 (2004) 1465-1475.

(39) L Li, B Julien, P Grenard, F Teixeira-Clerc, A Mallat, S Lotersztajn. Molecular mechanisms regulating the antifibrogenic protein heme-oxygenase-1 in human hepatic myofibroblasts, J.Hepatol. 41 (2004) 407-413.

(40) L Li, P Grenard, JT Nhieu, B Julien, A Mallat, A Habib, S Lotersztajn. Heme oxygenase-1 is an antifibrogenic protein in human hepatic myofibroblasts, Gastroenterology. 125 (2003) 460-469.

(41) D Han, N Hanawa, B Saberi, N Kaplowitz. Mechanisms of liver injury. III. Role of glutathione redox status in liver injury, Am.J.Physiol.Gastrointest.Liver Physiol. 291 (2006) G1-7.

(42) L Cesaratto, C Vascotto, S Calligaris, G Tell. The importance of redox state in liver damage, Ann.Hepatol. 3 (2004) 86-92.

(43) C Garcia-Ruiz, JC Fernandez-Checa. Redox regulation of hepatocyte apoptosis, J.Gastroenterol.Hepatol. 22 Suppl 1 (2007) S38-42.

(44) L Conde de la Rosa, MH Schoemaker, TE Vrenken, M Buist-Homan, R Havinga, PL Jansen, H Moshage. Superoxide anions and hydrogen peroxide induce hepatocyte death by different mechanisms: involvement of JNK and ERK MAP kinases, J.Hepatol. 44 (2006) 918-929.

Chapter 4

Overexpression of manganese superoxide dismutase reverses hepatic stellate cell activation

MH Tiebosch, F Haijer, F Braga Seganfredo, M Buist-Homan[1], H Moshage, KN Faber

Dept. Gastroenterology and Hepatology and [1]Dept. Laboratory medicine, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

In preparation

Abstract

Introduction: Liver fibrosis and cirrhosis are listed in the top 10 causes of death in the Western world. No effective therapy is available yet. Liver fibrosis is characterized by increased production of extracellular matrix (ECM) by hepatic stellate cells (HSCs). During fibrogenesis, HSCs transdifferentiate from a quiescent to a myofibroblast-like phenotype and produce excessive amounts of ECM. Activated HSCs have increased expression of Transforming Growth Factor β1 (TGF-β1), collagen type I and α-Smooth Muscle Actin (α-SMA). Superoxide anions, with mitochondria as a main source, have been implicated to promote HSC activation. Manganese superoxide dismutase (MnSOD) is an antioxidant enzyme in mitochondria. Previously, we found that MnSOD expression decreases dramatically upon HSC activation. Here, we studied the role of MnSOD in HSC activation.

Methods: Human MnSOD was overexpressed in activated rat hepatic stellate cells (aHSC) using an adenovirus (Ad-MnSOD). Messenger RNA levels of MnSOD and the stellate cell activation markers α-SMA, collagen type 1, TGF-β1 and PPAR-γ, were determined by qPCR. Protein expression was analyzed by Western blotting and immunofluorescence microscopy.

Results: Ad-MnSOD infection led to a transient increase in MnSOD expression in aHSC, which was accompanied by a decrease in the activation markers α-SMA (-80%), collagen type 1 (-70%) and TGF-β (-70%) and an increase in PPAR-γ (+ 2.4-fold), an adipogenic transcription factor. aHSC with increased MnSOD expression showed decreased cellular staining of collagen type 1 and α-SMA, of which also the typical filamentous structure was disturbed.

Conclusion: These data show that overexpression of MnSOD reduces the activation of HSCs *in vitro*. MnSOD is therefore a potential therapeutic target to treat liver fibrosis.

Introduction

Liver fibrosis and its end stage liver cirrhosis are severe disorders, with a high morbidity and mortality. The incidence of chronic liver diseases that progress to liver fibrosis and cirrhosis is increasing worldwide. To a large extent this is caused by the increased incidence of obesity and accompanying liver disorders like non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH).(1,2) Currently, there is no effective drug-based treatment available to arrest or reverse fibrosis and/or cirrhosis. Therefore, there is an urgent need to develop novel therapies to effectively treat patients with liver fibrosis.

Liver fibrosis is characterized by increased production of extracellular matrix (ECM) by myofibroblasts-like cells from different origins in the liver, such as the hepatic stellate cells (HSCs) and the portal myofibroblasts (PMFs).(3) Upon liver injury, these cells become activated and transdifferentiate into contractile and migratory cells that produce excessive amounts of extracellular matrix, in particular collagen type 1, leading to the formation of scar tissue. The increased expression of α-Smooth Muscle Actin (α-SMA) is a typical feature of hepatic myofibroblasts that supports the high contractility and mobility of these cells.

Most chronic liver diseases are accompanied by excessive production of reactive oxygen species (ROS) in the injured liver.(4,5) Whereas hepatocytes die in the presence of oxidative stress, hepatic stellate cells (HSCs) appear resistant to ROS-induced toxicity as they thrive in this harmful environment. Indeed, it has been speculated that ROS may in fact promote stellate cell activation and proliferation.(6-8) Several mechanisms may explain the resistance of HSCs to ROS-induced toxicity, including the expression of anti-oxidant enzymes. A number of anti-oxidants are required to efficiently detoxify ROS. First, superoxide dismutases convert superoxide into hydrogen peroxide, which is then further degraded by catalase and/or glutathione peroxidase. Three forms of superoxide dismutases exist; mitochondrial manganese superoxide dismutase (MnSOD, SOD 2), cytosolic copper-zinc superoxide dismutase (CuZnSOD, SOD 1) and extracellular dismutase (EcSOD, SOD3).(9) As mitochondrial oxidative phosphorylation is a major producer of ROS that may account for up to 90% of cellular ROS (10), it is believed that MnSOD is most important for detoxifying ROS in cells.

Previously, it has been shown that adenoviral-overexpression of MnSOD protects the rat liver in several animal models of liver injury, including alcoholic, cholestatic and

ischemia-reperfusion liver disease.(11-13) In apparent contradiction, we recently found that MnSOD mRNA and protein levels sharply drop upon *in vitro* activation of hepatic stellate cells, while CuZnSOD levels remain unchanged.(14) It is unclear whether HSC differentiation is causally related to MnSOD expression, or whether this is merely a consequence of HSC activation.

Therefore, the aim of this study was to increase MnSOD expression in activated hepatic stellate cells and study its effects on the activation state and to elucidate potential mechanisms involved in this process.

Methods & Materials

Cell isolations and culture conditions

Hepatic stellate cells (HSCs) were isolated from male Wistar rats (500-600 g) by pronase (Merck, Amsterdam, the Netherlands) and collagenase-P (Roche, Almere, The Netherlands) perfusion of the liver, followed by Nycodenz (Axis-Shield POC, Oslo, Norway) gradient (13% w/v) centrifugation as described previously.(15) Cells were then cultured in Iscove's Modified Dulbecco's Medium with Glutamax (Invitrogen, Breda, The Netherlands) supplemented with 20% heat-inactivated fetal calf serum (Invitrogen), 1 mmol/L sodium-pyruvate (Invitrogen), 1x MEM non-essential amino acids (Invitrogen), 50 μg/mL gentamycin (Invitrogen), 100 U/mL penicillin (Lonza, Vervier, Belgium), 10 μg/mL streptomycin (Lonza), 250 ng/mL fungizone (Lonza) in a humidified atmosphere containing 5% CO_2 at 37 °C. Primary HSC cultures were allowed to culture-activate in 7 days, then passaged via trypsinization. The cells were used for experiments at passage 1, after allowing attachment in 6-wells plates. All experiments were performed in serum-free conditions unless described otherwise.

Adenoviral constructs and infection of activated hepatic stellate cells with adenoviruses

HSCs were transfected with a recombinant adenovirus containing the human MnSOD cDNA (AdMnSOD). A LacZ/β-gal containing adenovirus was used as a control virus. The AdMnSOD adenovirus was a kind gift of prof. T. O'Brien, National University of Ireland, Galway, Ireland and has been described before.(16) Optimal duration and multiplicity of infection (MOI) were determined.

RNA isolation, RT-PCR and quantitative real time PCR

RNA was isolated using Tri-reagent (Sigma-Aldrich) according to the manufacturer's instructions. Reverse transcription was performed on 1μg RNA using random nanomers (Sigma-Aldrich) in a final volume of 50 µL. Reverse transcription was performed in three steps: 10 minutes at 25°C, 1 hour at 37°C and 5 minutes at 95°C. Real time detection was performed on the ABI PRISM 7700 (PE Applied biosystems) using the Taqman protocol. This protocol includes an initiation of 10 min at 95°C, followed by 40 cycles (15 seconds at 95°C, and 1 minute at 60°C). mRNA levels of 18S were used as an internal control. Each sample was analyzed in duplicate. Relative gene expressions were calculated using the ΔΔCt method. The primers (Invitrogen) and probes (Eurogentec) used, are described in Table 1. To determine human MnSOD mRNA expression produced as a consequence of adenoviral MnSOD infection, we used a Taqman Assay on Demand from Invitrogen, however we found that this also detected rat MnSOD expression.

Table 1 Sequences of primers and probes used for quantitative PCR analysis

Gene		Primers
18S rat	Sense	5′-CGG CTA CCA CAT CCA AGG A-3′
	Anti-sense	5′-CCA ATT ACA GGG CCT CGA AA-3′
	Probe	5′-CGC GCA AAT TAC CCA CTC CCG A -3′
αSMA rat	Sense	5′-GCC AGT CGC CAT CAG GAA C-3′
	Anti-sense	5′-CAC ACC AGA GCT GTG CTG TCT T-3′
	Probe	5′-CTT CAC ACA TAG CTG GAG CAG CTT CTC GA-3′
Catalase rat	Sense	5′-GGA TTA TGG CCT CCG AGA TCT-3′
	Anti-sense	5′-ACC TTG GTC AGG TCA AAT GGA T-3′
	Probe	5′-ATG CCA TCG CCA GTG GCA ATT ACC-3′
Collagen type 1 rat	Sense	5′-TGG TGA ACG TGG TGT ACA AGG T-3′
	Anti-sense	5′-CAG TAT CAC CCT TGG CAC CAT-3′
	Probe	5′-TCC TGC TGG TCC CCG AGG AAA CA-3′
CuZnSOD rat	Sense	5′-CAG GAC CTC ATT TTA ATC CTC ACT C-3′
	Anti-sense	5′-GTC TCC AAC ATG CCT CTC TTC A-3′
	Probe	5′-CCG CTG GAC CGC CAT GTT TCT T-3′
GPx1 rat	Sense	5′-GGACATCAGGAGAATGGCAAGA-3′
	Anti-sense	5′-CGCACTTCTCAAACAATGTAAAGTTG-3′
	Probe	5′-TTCCCTCAAGTATGTCCGACCCGGTG-3′
PPAR-γ rat	Sense	5′-CAC AAT GCC ATC AGG TTT GG-3′
	Anti-sense	5′-GCT GGT CGA TAT CAC TGG AGA TC-3′
	Probe	5′-CCA ACA GCT TCT CCT TCT CGG CCT G-3′
TGFβ rat	Sense	5′-GGG CTA CCA TGC CAA CTT CTG-3′
	Anti-sense	5′-GAG GGC AAG GAC CTT GCT GTA-3′
	Probe	5′-CCT GCC CCT ACA TTT GGA GCC TGG A-3′

MitoTracker

In order to confirm the targeting of the recombinant MnSOD protein to the mitochondria, we incubated the cells at 37°C with MitoTracker® Red CMXROS (Life technologies) at an end concentration of 50 nmol/L. After allowing 30 minutes for the probe to enter the cells, cells were fixed using 4% paraformaldehyde/PBS and were processed for immunofluorescence microscopy.

Immunofluorescence microscopy

(17)Activated HSCs were cultured on glass coverslips. After adenoviral transfection, cells were fixed using 4% paraformaldehyde/PBS. Prior to staining, cells were permeabilized with 1% Triton-X100 for 5 minutes. Non-specific antibody binding sites were blocked for 30 min in 0.5% BSA/PBS. Subsequently, cells were incubated with primary antibodies (MnSOD: Stressgen, rabbit polyclonal, dilution 1:400 in 0.5% BSA/PBS; α-SMA: Sigma, mouse monoclonal, dilution 1:500 in 0.5% BSA/PBS; collagen 1α1: Southern Biotech, goat polyclonal, dilution 1:30 in 0.5% BSA/PBS) for 1-2 hours at room temperature. Alexa fluorophores (Molecular probes, diluted 1:500) were used as secondary antibodies. Finally, coverslips were mounted in fluorescence mounting medium S3023 containing DAPI for nuclear staining (DAKO). The slides were visualized using a Zeiss 410 inverted laser scan microscope.

Statistical analysis

All results are presented as the mean of at least three independent experiments using cells from different rats ± standard deviation. Statistical analysis of the results was performed using GraphPad Prism 5 for Windows. A student t-test or two-way ANOVA followed by Bonferroni's multiple comparison tests were used to determine significant differences between the groups. A p-value <0.05 was considered statistically significant.

Results

MnSOD expression is induced upon infection of hepatic stellate cells

First, we optimized the infection procedure for culture-activated HSC using the MnSOD-expressing adenovirus. Only at a high multiplicity of infection (MOI) of 1,000 we detected a significant and reproducible increase in MnSOD transcript levels **(Figure**

1A and 1B), which was not observed in Ad-LacZ-infected control aHSC (comparison to un-infected control cells is not shown). The Ad-MnSOD-mediated increase in MnSOD mRNA levels was time-dependent and transient, peaking at 72 h after which it sharply dropped **(Figure 1C)**. MnSOD protein levels, however, were only clearly increased after 96h of infection **(Figure 1D)**, indicating a significant delay between peak RNA levels and production of the corresponding protein. In the following experiments, effects of HSC activation were therefore analyzed 96 h after infection.

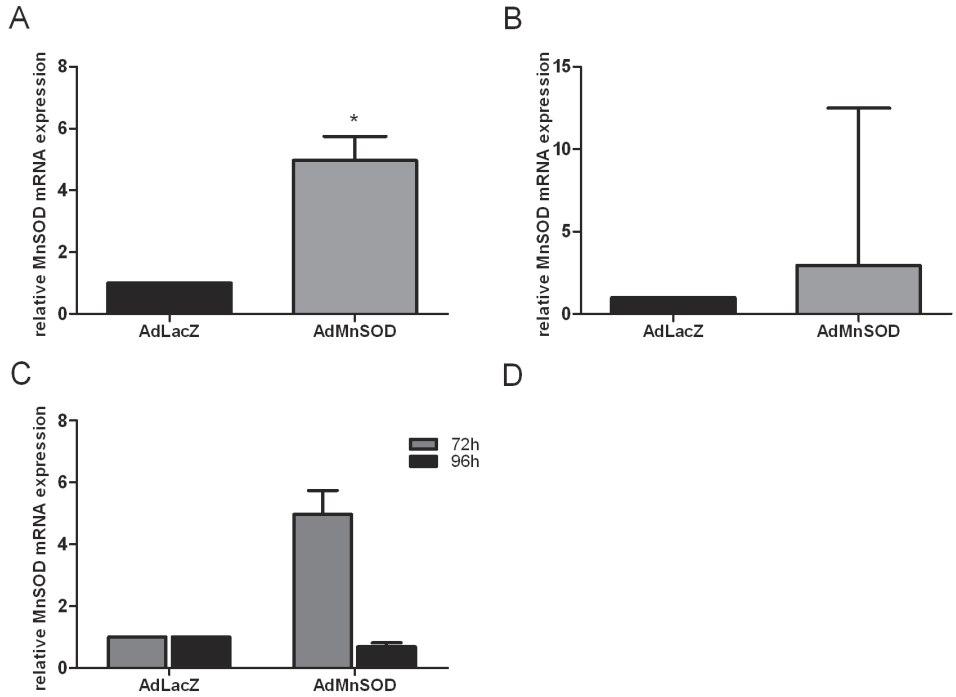


Figure 1. MnSOD expression is induced upon infection of hepatic stellate cells
A: After 72h of infection of aHSC with Ad-MnSOD (MOI 1000), MnSOD mRNA levels were 5-fold induced compared to Ad-LacZ-infected cells. * $p<0.05$ **B:** After 72h of Ad-MnSOD infection with a lower multiplicity of infection (MOI 500) there is hardly any induction of MnSOD mRNA levels. **C:** MnSOD mRNA levels are transiently increased with a maximum after 72h of infection. **D:** Protein levels of MnSOD are increased after 96h of Ad-MnSOD infection (MOI 1000).

Artificial overexpression of MnSOD reverses hepatic stellate cell activation

Adenoviral-mediated overexpression of MnSOD led to a strong reduction in mRNA levels of the typical HSC activation markers α-SMA (80% reduction), collagen1a1 (70% reduction and TGF-β (70% reduction) compared to Ad-LacZ-infected control aHSC

(Figure 2A-C). In contrast, transcript levels for the marker for quiescent HSC, the nuclear receptor peroxisome proliferator-activated gamma (PPAR-y), were significantly increased (2.4-fold; **Figure 2D**).

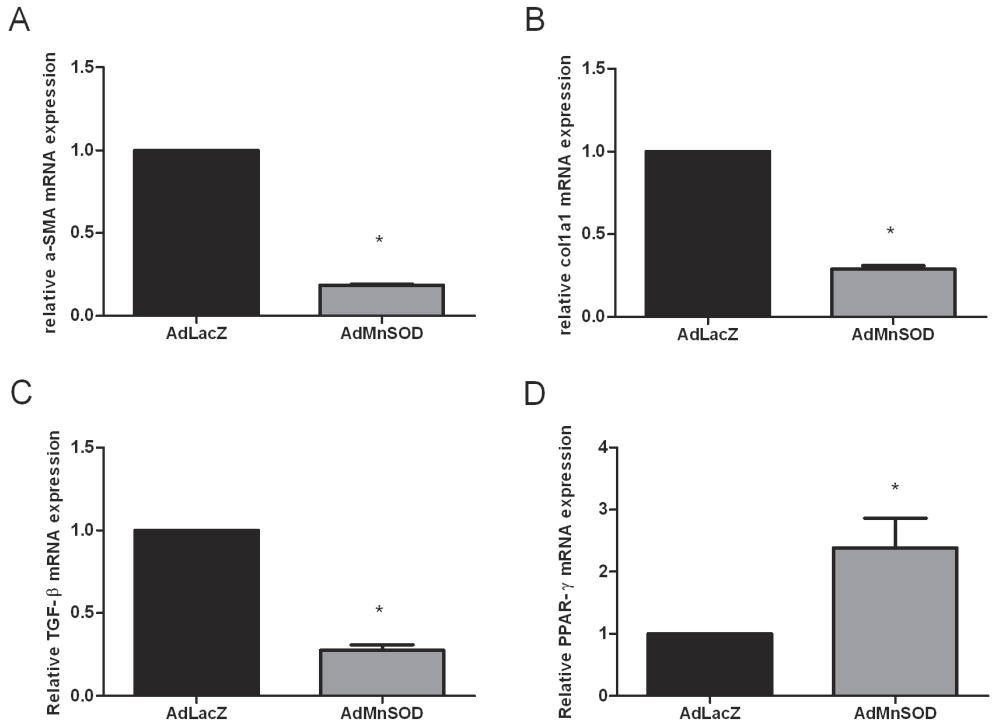


Figure 2. MnSOD induction results in a reversal to the quiescent phenotype of HSC
Ad-MnSOD infection (MOI 1000) during 96h of aHSC caused an 80% reduction of α-smooth muscle actin (**A**), a 70% reduction of collagen 1α1 (**B**) and a 70% reduction of transforming growth factor-β (**C**) mRNA levels compared to Ad-LacZ infected aHSC. **D:** Upon MnSOD induction mRNA levels of peroxisome proliferator-activated gamma were increased 2.4 fold compared to AdLacZ-infected aHSC. * p<0,05.

Immunofluorescence microscopy

Next, we analyzed the Ad-MnSOD-infected aHSC by immunofluorescence microscopy in order to determine the activation status of HSC at the cellular level. Approximately 50% of the cells showed clearly increased levels of MnSOD that colocalized with Mitotracker, indicating proper targeting of the recombinant protein in HSCs (Figure 3A). The HSC with increased MnSOD expression appeared smaller in size. Double staining for intracellular levels of collagen1α1 showed clearly reduced levels in aHSC expressing high levels of

MnSOD. (Figure 3B). A similar effect was observed for α-SMA staining, which revealed that the cells with higher MnSOD levels show a decreased α-SMA expression. Moreover, the typical filamentous pattern of α-SMA in aHSC is largely absent. (Figure 3C)

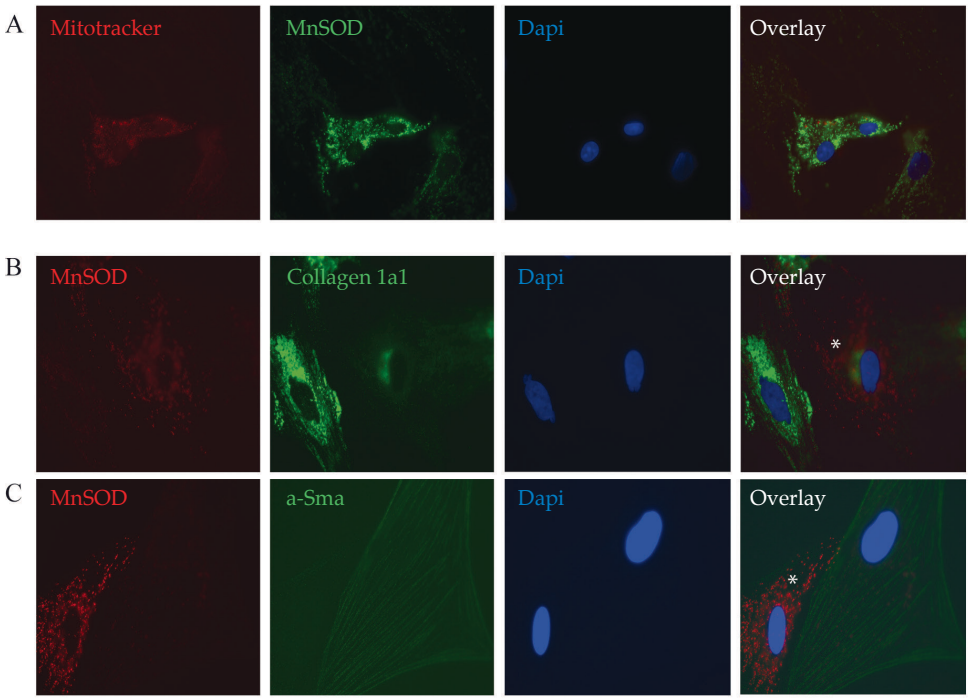


Figure 3. Upon induction of MnSOD, the phenotype of HSC changes
A: Approximately 50% of the cells showed clearly increased levels of MnSOD (in green) that colocalized with Mitotracker (in red). **B:** Cells with increased MnSOD expression levels (*, in red) show reduced intracellular collagen1α1 expression (in green) compared to cells with a lower expression level of MnSOD. All aHSC were infected for 96h with Ad-MnSOD (MOI 1000). **C**: Cells with increased MnSOD expression (*, in red) show reduced α-SMA (green) staining, with a disrupted filamentous structure compared to cells with a lower expression level of MnSOD. Magnification of all photos: 630x.

Effect on expression of other antioxidant enzymes

Artificial overexpression of MnSOD may change the expression of other antioxidant enzymes. However, CuZnSOD expression levels were not significantly different between Ad-LacZ and Ad-MnSOD infected aHSC. The increased levels of MnSOD potentially lead to increased conversion of superoxides to hydrogen peroxide and thus we analyzed the mRNA levels of antioxidants that are responsible for the detoxification of H_2O_2. Ninety six (96) hours post-infection, transcript levels of catalase and glutathione peroxidase 1

(GPx1) were slightly reduced in Ad-MnSOD treated HSC compared to Ad-LacZ-infected control cells **(Figure 4A-B)**. However, only the difference in Gpx1 expression levels between Ad-MnSOD- and Ad-LacZ-infected aHSC reached statistical significance.

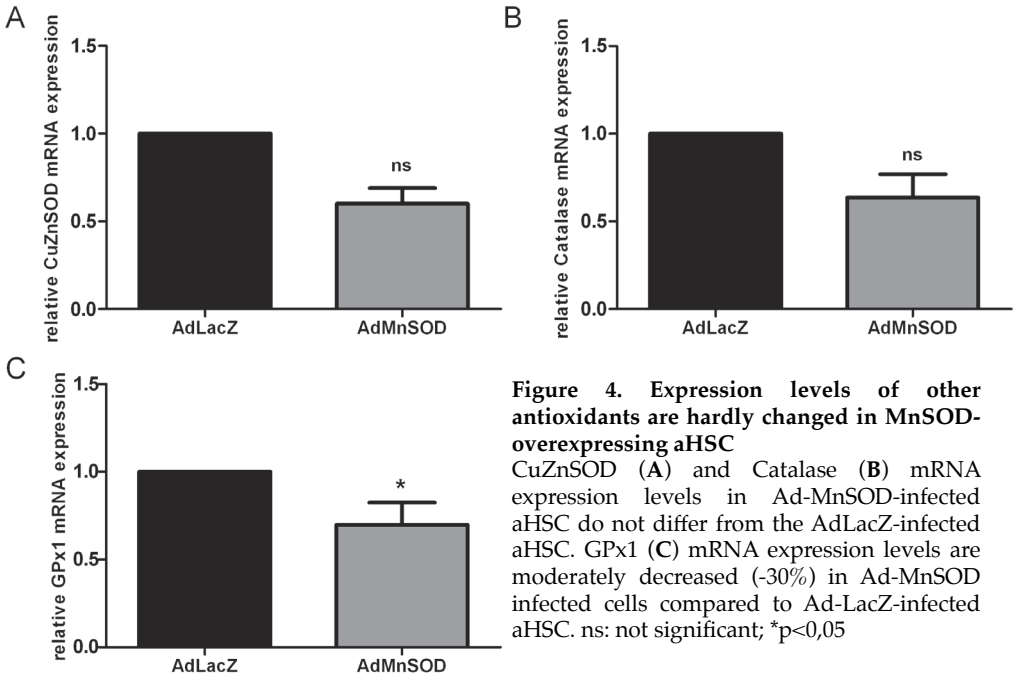


Figure 4. Expression levels of other antioxidants are hardly changed in MnSOD-overexpressing aHSC
CuZnSOD (**A**) and Catalase (**B**) mRNA expression levels in Ad-MnSOD-infected aHSC do not differ from the AdLacZ-infected aHSC. GPx1 (**C**) mRNA expression levels are moderately decreased (-30%) in Ad-MnSOD infected cells compared to Ad-LacZ-infected aHSC. ns: not significant; *p<0,05

Discussion

In this study, we show that recombinant expression of mitochondrial MnSOD in activated hepatic stellate cells reduces their activation state, as indicated by reduced collagen1α1 and α-SMA expression. MnSOD levels in aHSC thus directly control their fibrogenic potential and increasing MnSOD expression in chronic liver disease may therefore reduce liver fibrosis.

Concomitant with the reduction of collagen1α1 and α-SMA mRNA levels, PPAR-γ expression was increased in MnSOD-overexpressing aHSC. PPAR-γ is a central factor in HSC transdifferentiation and its expression is strongly suppressed in aHSC compared to quiescent HSC (qHSC).(18,19) As PPAR-γ is an adipogenic transcription factor, it controls adipocyte specific genes, expressed by the quiescent hepatic stellate cells.

(20,21) Our data suggest that PPAR-γ expression is induced by MnSOD. From previous studies, it is known that PPAR-γ expression is influenced by oxidative stress.(22,23) As we speculate that induction of MnSOD expression influences the oxidative stress levels in hepatic stellate cells, PPAR-γ expression could be influenced in this way. However, another possibility is that MnSOD triggers quiescence through other pathways, by which PPAR-γ is induced. The human *SOD2* gene contains a PPAR-γ binding motif (PPRE) in the promoter region; however, this was shown to suppress MnSOD expression.(24) The promoter of rat *SOD2* also contains PPRE elements, though they are not completely homologous to those found in the human promoter. Still, it is likely that PPAR-γ also influences rat MnSOD expression. From our experiments, we conclude that PPAR-γ and MnSOD stimulate each other in a positive direction, since both MnSOD and PPAR-γ decrease upon activation of HSC and increase again upon artificial induction of quiescence. To support this hypothesis, MnSOD expression levels should be assessed upon stimulation of aHSC with PPAR-γ agonists.

Along with MnSOD induction, a decrease in (mitochondrial) superoxides is expected, and a potential increase of hydrogen peroxide (since catalase and GPx1 mRNA levels are slightly decreased). This switch in ROS can possibly lead to altered physiological signaling, resulting in a reduced activation state of the stellate cells. Previously, it was shown that superoxide production is linked to proliferation and that SOD mimetics were able to block this.(25) Since activated hepatic stellate cells are highly proliferative, decreased superoxide levels due to the induced MnSOD expression may lead to reduced HSC activation. Furthermore, it has been described that MnSOD levels induce transition between quiescent and proliferative growth in embryonic fibroblasts; high levels of MnSOD and low superoxide levels are associated with quiescence whereas low levels of MnSOD with high superoxide levels are associated with proliferation.(26) Although different cells were used, the similarities concerning the transdifferentiation from quiescent to proliferative cells are notable, and therefore this mechanism could also be true for hepatic stellate cells. Future experiments to investigate these mechanisms further should include assessment of superoxide and hydrogen peroxide levels as well as proliferation assays.

It is suggested that HSC activation is accompanied with a major change in mitochondrial function, since they switch from oxidative phosphorylation to glycolysis, while the number of mitochondria is retained (known as the Warburg effect).(27) Moreover, mild mitochondrial uncoupling leads to reduced activation of HSC.(28)

Increasing MnSOD expression, leading to reduced mitochondrial superoxides, could lead to reduced mitochondrial stress and possibly increase oxidative phosphorylation. Induction of MnSOD was shown to reduce the growth rate of multiple types of cancer cells (29), as well as inhibit lipid peroxidation. As activated stellate cells share many similarities with cancer cells, such as proliferation and a change in metabolism (Warburg effect), we hypothesize that the protective mechanism of MnSOD induction is similar in HSCs and cancer cells, and possibly is associated with improved mitochondrial function. In order to get more evidence for this hypothesis, future experiments should include measurements of oxidative phosphorylation in aHSC with or without induction of MnSOD levels.

Previous studies showed that *in vivo* overexpression of MnSOD was protective in both cholestatic liver injury and alcohol liver disease, probably through a reduction of mitochondrial oxidative stress.(11,12) However, in these *in vivo* experiments, adenoviral gene delivery most likely is targeted to the hepatocytes, the main liver cell type. Therefore, the effects described in these studies are unlikely related to the repression of HSC activation. Apparently, there is dual effect of MnSOD, both protecting hepatocytes from oxidative stress and preventing hepatic stellate cells from activating.

In summary, we conclude that MnSOD induction in activated rat hepatic stellate cells reverses stellate cell activation. This provides further evidence to the concept that oxidative stress, especially mitochondrial reactive oxygen species, plays an important role in liver fibrosis.

Acknowledgements

Microscopic fluorescence imaging was performed at the UMCG Microscopy & Imaging Center (UMIC), which is supported by the Netherlands Organisation for Health Research and Development (ZonMW grant 40-00506-98-9021).

References

(**1**) Clark JM. The epidemiology of nonalcoholic fatty liver disease in adults. J Clin Gastroenterol 2006 Mar;40 Suppl 1:S5-10.

(**2**) Marchesini G, Bugianesi E, Forlani G, Cerrelli F, Lenzi M, Manini R, et al. Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. Hepatology 2003 Apr;37(4):917-923.

(**3**) Lemoinne S, Cadoret A, El Mourabit H, Thabut D, Housset C. Origins and functions of liver myofibroblasts. Biochim Biophys Acta 2013 Jul;1832(7):948-954.

(**4**) Poli G. Pathogenesis of liver fibrosis: role of oxidative stress. Mol Aspects Med 2000 Jun;21(3):49-98.

(**5**) Muriel P. Role of free radicals in liver diseases. Hepatol Int 2009 Nov 26.

(**6**) Galli A, Svegliati-Baroni G, Ceni E, Milani S, Ridolfi F, Salzano R, et al. Oxidative stress stimulates proliferation and invasiveness of hepatic stellate cells via a MMP2-mediated mechanism. Hepatology 2005 May;41(5):1074-1084.

(**7**) Nieto N, Friedman SL, Cederbaum AI. Stimulation and proliferation of primary rat hepatic stellate cells by cytochrome P450 2E1-derived reactive oxygen species. Hepatology 2002 Jan;35(1):62-73.

(**8**) Guimaraes EL, Franceschi MF, Grivicich I, Dal-Pizzol F, Moreira JC, Guaragna RM, et al. Relationship between oxidative stress levels and activation state on a hepatic stellate cell line. Liver Int 2006 May;26(4):477-485.

(**9**) Miao L, St Clair DK. Regulation of superoxide dismutase genes: implications in disease. Free Radic Biol Med 2009 Aug 15;47(4):344-356.

(**10**) Venditti P, Di Stefano L, Di Meo S. Mitochondrial metabolism of reactive oxygen species. Mitochondrion 2013 Mar;13(2):71-82.

(**11**) Zhong Z, Froh M, Wheeler MD, Smutney O, Lehmann TG, Thurman RG. Viral gene delivery of superoxide dismutase attenuates experimental cholestasis-induced liver fibrosis in the rat. Gene Ther 2002 Feb;9(3):183-191.

(**12**) Wheeler MD, Nakagami M, Bradford BU, Uesugi T, Mason RP, Connor HD, et al. Overexpression of manganese superoxide dismutase prevents alcohol-induced liver injury in the rat. J Biol Chem 2001 Sep 28;276(39):36664-36672.

(**13**) Wheeler MD, Katuna M, Smutney OM, Froh M, Dikalova A, Mason RP, et al. Comparison of the effect of adenoviral delivery of three superoxide dismutase genes against hepatic ischemia-reperfusion injury. Hum Gene Ther 2001 Dec 10;12(18):2167-2177.

(**14**) Dunning S, Ur Rehman A, Tiebosch MH, Hannivoort RA, Haijer FW, Woudenberg J, et al. Glutathione and antioxidant enzymes serve complementary roles in protecting activated hepatic stellate cells against hydrogen peroxide-induced cell death. Biochim Biophys Acta 2013 Jul 16.

(**15**) Moshage H, Casini A, Lieber CS. Acetaldehyde selectively stimulates collagen production in cultured rat liver fat-storing cells but not in hepatocytes. Hepatology 1990 Sep;12(3 Pt 1):511-518.

(**16**) Duffy AM, O'Brien T, McMahon JM. Generation of antioxidant adenovirus gene transfer vectors encoding CuZnSOD, MnSOD, and catalase. Methods Mol Biol 2010;594:381-393.

(**17**) Conde de la Rosa L, Schoemaker MH, Vrenken TE, Buist-Homan M, Havinga R, Jansen PL, et al. Superoxide anions and hydrogen peroxide induce hepatocyte death by different mechanisms: involvement of JNK and ERK MAP kinases. J Hepatol 2006 May;44(5):918-929.

(**18**) Kisseleva T, Brenner DA. Inactivation of myofibroblasts during regression of liver fibrosis. Cell Cycle 2013 Feb 1;12(3):381-382.

(**19**) Yu J, Zhang S, Chu ES, Go MY, Lau RH, Zhao J, et al. Peroxisome proliferator-activated receptors gamma reverses hepatic nutritional fibrosis in mice and suppresses activation of hepatic stellate cells in vitro. Int J Biochem Cell Biol 2010 Jun;42(6):948-957.

(**20**) MacDougald OA, Lane MD. Transcriptional regulation of gene expression during adipocyte differentiation. Annu Rev Biochem 1995;64:345-373.

(**21**) Morrison RF, Farmer SR. Hormonal signaling and transcriptional control of adipocyte differentiation. J Nutr 2000 Dec;130(12):3116S-3121S.

(**22**) Blanquicett C, Kang BY, Ritzenthaler JD, Jones DP, Hart CM. Oxidative stress modulates PPAR gamma in vascular endothelial cells. Free Radic Biol Med 2010 Jun 15;48(12):1618-1625.

(**23**) Tickner J, Fan LM, Du J, Meijles D, Li JM. Nox2-derived ROS in PPARgamma signaling and cell-cycle progression of lung alveolar epithelial cells. Free Radic Biol Med 2011 Aug 1;51(3):763-772.

(**24**) Venkatachalam G, Kumar AP, Yue LS, Pervaiz S, Clement MV, Sakharkar MK. Computational identification and experimental validation of PPRE motifs in NHE1 and MnSOD genes of human. BMC Genomics 2009 Dec 3;10 Suppl 3:S5-2164-10-S3-S5.

(**25**) Yang JQ, Buettner GR, Domann FE, Li Q, Engelhardt JF, Weydert CD, et al. v-Ha-ras mitogenic signaling through superoxide and derived reactive oxygen species. Mol Carcinog 2002 Apr;33(4):206-218.

(**26**) Sarsour EH, Venkataraman S, Kalen AL, Oberley LW, Goswami PC. Manganese superoxide dismutase activity regulates transitions between quiescent and proliferative growth. Aging Cell 2008 Jun;7(3):405-417.

(**27**) Chen Y, Choi SS, Michelotti GA, Chan IS, Swiderska-Syn M, Karaca GF, et al. Hedgehog controls hepatic stellate cell fate by regulating metabolism. Gastroenterology 2012 Nov;143(5):1319-29.e1-11.

(**28**) Guimaraes EL, Best J, Dolle L, Najimi M, Sokal E, van Grunsven LA. Mitochondrial uncouplers inhibit hepatic stellate cell activation. BMC Gastroenterol 2012 Jun 11;12:68-230X-12-68.

(**29**) Oberley LW. Mechanism of the tumor suppressive effect of MnSOD overexpression. Biomed Pharmacother 2005 May;59(4):143-148.

Chapter 5

Caffeine has direct and indirect antifibrotic properties *in vitro*

Tiebosch MH, Naspolini N, Buist-Homan M[1], Henning R[2], Arese M[3], Faber KN, Moshage H

Dept. Gastroenterology and Hepatology and [1]Dept. Laboratory Medicine [2]Dept. of Clinical Pharmacology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands. [3]Pontifical Universidad de Chile, Santiago, Chile

In preparation

Abstract

Introduction Caffeine is known from previous epidemiological studies to prevent the onset as well as aggravation of liver disease, including liver fibrosis. This was confirmed in *in vivo* studies in several models of liver disease, such as the cholestatic model of bile duct ligation, and the cytotoxic models CCl_4 and TAA. It has been suggested that the antifibrotic and protective effects of caffeine are due to its metabolites, such as dimethylxanthines. However, a direct effect of caffeine has not been excluded and has never been investigated.

Aim of the study was to investigate the effect of caffeine and its main metabolite on several cell types involved in liver fibrogenesis.

Methods/results Primary rat hepatic stellate cells and portal myofibroblasts were culture-activated and treated with either caffeine, its main metabolite 1,7-dimethylxanthine (1,7-DMX) or the combination of these two. Markers of myofibroblast activation, such as alpha-smooth muscle actin (α-SMA), and collagen1 α 1, were reduced by both caffeine and 1,7-DMX, both at the mRNA (qPCR) and protein (Western blot, immunofluorescence) level up to 60% at a concentration of 20mmol/L of caffeine after 72h. The combination of caffeine and 1,7-DMX was able to reduce α-SMA and collagen1 α 1 mRNA levels even further. In addition, caffeine was able to reduce the proliferative capacity of the stellate cells. Since caffeine is to be metabolized by CYP1A2, we determined the expression levels in liver cells. It was hardly expressed in any cell type other than hepatocytes. Caffeine and 1,7-DMX also have anti-inflammatory properties, in primary Kupffer cells pre-incubation resulted in reduced LPS-induced TNF-α mRNA levels. In primary hepatocytes, pre-incubation with 1,7-DMX resulted in reduced cytokine mixture-induced iNOS mRNA levels.

Conclusion Caffeine is able to reduce activation as well as proliferation of stellate cells *in vitro,* independent of its main metabolite 1,7-DMX. However, in combination their effects are complementary. Moreover, caffeine and 1,7-DMX have anti-inflammatory properties. These results may explain the protective effects of caffeine on liver fibrogenesis.

Introduction

Liver diseases can have a diverse etiology. Well known causes are hepatitis B or C infection, non-alcoholic fatty liver disease (NAFLD) associated with obesity, and alcohol abuse. Chronic liver disease can lead to liver fibrosis and its end stage liver cirrhosis. These are severe disorders, with high morbidity and mortality. Currently, no effective drug-based treatment is available to reverse or arrest fibrosis and/or cirrhosis.

From epidemiological studies it is known that patients with liver fibrosis or cirrhosis caused by NAFLD and hepatitis C virus infection benefit from coffee consumption compared to non-coffee drinkers: the risk of liver disease progression was reduced as evaluated by biopsies and biochemical parameters.(1-4) The amount of coffee needed to achieve this risk reduction is unclear, however one study reported a consumption of more than two units a day to obtain a beneficial effect.(3) Explaining the observed protective effects, an anti-inflammatory mechanism was suggested, as well as reduced risk for diabetes mellitus type 2. Whether the beneficial effect observed from coffee can be attributed to caffeine is under debate: some studies do not find a negative (protective) correlation between caffeine and fibrosis (4), while others observed a protective effect of caffeine when comparing caffeinated and decaffeinated coffee (3). Several studies however, could not show a beneficial effect of black tea or other caffeinated beverages. (1,2)

To confirm the observed beneficial effects in epidemiological studies, several studies were performed in *in vivo* models of liver disease. In cytotoxic liver disease induced by carbon tetrachloride (CCL_4) grain coffee showed antifibrotic effects on biochemical and histological level.(5) Also lipid peroxidation was reduced which indicates an anti-oxidative effect. In another cytotoxic liver disease model, induced by thioacetamide (TAA), the effect of caffeine as an ingredient of coffee was studied. Conventional coffee, and to a lesser extent decaffeinated coffee and caffeine separately reduced ALT (alanine transaminase, serum marker for hepatocyte necrosis) and histological scores for fibrosis and inflammation induced by TAA. However, only conventional coffee and caffeine induced a reduction of PCNA-S phase positive cells (proliferating cellular nuclear antigen, a marker for proliferation) expression, while only conventional coffee reduced metalloproteinase-2 (MMP2, a marker for fibrosis) activity. Thus, caffeine seems to have an independent effect on fibrosis, although the main effect is accomplished using the whole coffee extract.(6) However, in another study only caffeine injections reduced

steatohepatitis in a model of alcoholic liver disease.(7) Moreover in the same study, Kupffer cells isolated from mice with alcoholic liver disease treated with caffeine were less activated upon endotoxin (LPS)-induced activation. This supports the hypothesis that caffeine has independent protective effects on liver disease.

In cholestatic liver disease induced by bile duct ligation (BDL), caffeine and its main metabolite 1,7-dimethylxanthine (1,7-DMX) proved to be antifibrotic as well as to reduce lipid peroxidation.(8)

Few *in vitro* studies with caffeine and/or its metabolites have been performed on this subject, and they focused mainly on hepatic stellate cells. Upon chronic injury these cells transdifferentiate into myofibroblasts, producing extracellular matrix, the major hallmark of fibrosis. Caffeine and 1,7-DMX were found to reduce expression of profibrotic connective tissue growth factor (CTGF), whereas hepatocytes were not damaged by caffeine or 1,7-DMX.(8) Next to hepatic stellate cells, also portal myofibroblasts can contribute to extracellular matrix production.

As fibrogenesis is accompanied by inflammation, it is also relevant to investigate the effect of caffeine (and its metabolites) on inflammatory cells such as Kupffer cells, the resident macrophages of the liver. Finally, caffeine (or its metabolites) can have effects on hepatocytes, the most predominant liver cell type. Hepatocytes are exposed to all incoming compounds from the gut via the portal circulation, including damaging stimuli and microbial/viral agents, possibly leading to hepatocyte death. Dying hepatocytes contribute to liver injury via different mechanisms: hepatocytes are the functional liver cell type and their death eventually leads to liver failure, but dying hepatocytes can also aggravate liver inflammation, by attracting inflammatory cells.

In order to further clarify the protective effect of caffeine observed in epidemiological studies, and to further determine the *in vitro* mechanism of these protective effects, we investigated whether caffeine or its major metabolite has antifibrotic, anti-inflammatory or other protective effects on hepatic stellate cells, portal myofibroblasts, Kupffer cells and hepatocytes.

Methods

Animals

Specified pathogen-free male Wistar rats were purchased from Charles River Laboratories Inc. (Wilmington, MA, USA). Animals were kept under standard laboratory conditions with free access to standard laboratory chow and water. All experiments were performed in accordance with the guidelines of the local Committee for Care and Use of laboratory animals.

Hepatic stellate isolation

Hepatic stellate cells (HSCs) were isolated from male Wistar rats (500-600 g) by pronase (Merck, Amsterdam, the Netherlands) and collagenase-P (Roche, Almere, The Netherlands) perfusion of the liver, followed by Nycodenz (Axis-Shield POC, Oslo, Norway) gradient (13% w/v) centrifugation as described previously.(9) Hepatic stellate cells were cultured in Iscove's Modified Dulbecco's Medium with Glutamax (Invitrogen, Breda, The Netherlands) supplemented with 20% heat-inactivated fetal calf serum (Invitrogen), 1 mmol/L sodium-pyruvate (Invitrogen), 1x MEM non essential amino acids (Invitrogen), 50 μg/mL gentamycin (Invitrogen), 100 U/mL penicillin (Lonza, Vervier, Belgium), 10 μg/mL streptomycin (Lonza), 250 ng/mL fungizone (Lonza) in a humidified atmosphere containing 5% CO_2 at 37 °C. Purity was checked by autofluorescence and phenotypical appearance.

Hepatocyte isolation

Hepatocytes were isolated from male Wistar rats (220-250g) by a two-step collagenase I perfusion as described previously.(10) Cell viability was greater than 85% as determined by Trypan Blue exclusion. After isolation, 112500 cells per cm^2 were plated on Vitrogen® (Cohesion Technologies Inc., Palo Alto, CA, USA) coated plates in William's E medium (Invitrogen, Breda, The Netherlands) supplemented with 50 μg/mL gentamycin (Invitrogen) and penicillin–streptomycin–fungizone (Lonza, Verviers, Belgium). During the attachment period (4 h) 50 nmol/L dexamethasone (Department of Pharmacy UMCG, Groningen, The Netherlands) and 5% fetal calf serum (Invitrogen) were added to the medium. Cells were cultured in a humidified incubator at 37 °C and 5% CO_2.

Portal myofibroblast isolation

Portal myofibroblasts were isolated from the residues of the portal tree after hepatocyte isolation (see above) as described earlier.(11) In short, the residues of the intrahepatic bile duct were minced and subsequently incubated for 30 min at 37°C in a digestion solution containing collagenase P (0,66%), pronase (0,055%), DNase (0,006%), fetal calf serum (3%), bovine serum albumin (0,1%), Hepes (10mmol/L) and penicillin-streptomycin-fungizone (100000 IU-100mg/L). Next, the solution was filtered and the incubation step was repeated with a newly made digestion solution. Finally, the residues were incubated for 30 min at 37°C in a digestion solution where pronase was replaced by hyaluronidase (0,036%). After filtering and centrifugation to collect the cells, considered portal myofibroblasts, they were cultured in a humidified incubator at 37 °C and 5% CO_2, and were allowed to proliferate until confluency was reached. The portal myofibroblasts were cultured in the same medium as hepatic stellate cells (see above): IMDM containing 20% FCS.

Kupffer cell isolation

Kupffer cells were isolated from the non-parenchymal cell fraction obtained after hepatocyte isolation. The Kupffer cells in this fraction were isolated using a Percoll density cushion, as described previously.(12) The cells were allowed to adhere to the culture plates for 30 min in HBSS containing Mg^{2+},$Ca^{2+}$and 10% FCS. Next, the contaminating hepatocytes were removed by washing and cells were cultured overnight in RPMI containing 10% FCS in a humidified incubator at 37 °C and 5% CO_2. Purity of the cell population was checked by ED-1 staining.

Experimental design

Activated hepatic stellate cells were used at passage 1, after 7 days of culture. Then the cells were incubated with the indicated concentrations of caffeine and/or 1,7-DMX. At the indicated time points the cells were harvested for the different assays.

Portal myofibroblasts were used at passage 2 to 3. The cells were incubated with the indicated concentrations of caffeine and/or 1,7-DMX and at the indicated time points the cells were harvested for the different assays. Portal myofibroblasts were cultured in serum containing medium throughout the experiments.

Hepatocytes were pre-incubated for 0.5 hr with 1,7-DMX at the indicated concentrations. Next, the cells were incubated with a cytokine mixture composed

of interleukin 1β (IL-1β; 10 ng/ml), tumor necrosis factor-α (TNFα; 10 ng/ml) and interferon-γ (IFNy; 10 ng/ml). At the indicated time points cells were harvested for the different assays.

Kupffer cells were pre-incubated for 0.5 hr with either 1,7-DMX or caffeine at the indicated concentrations. After the pre-incubation the cells were incubated with LPS (0.1μg/mL) for 5h. At the indicated time points cells were harvested for the different assays.

All the experiments were performed in serum free medium, unless indicated otherwise.

RNA isolation, RT-PCR and quantitative real time PCR

RNA was isolated using Tri-reagent (Sigma-Aldrich) according to the manufacturer's instructions. Reverse transcription was performed on 1μg RNA using random nanomers (Sigma-Aldrich) in a final volume of 50 μL. Reverse transcription was performed in three steps: 10 minutes at 25°C, 1 hour at 37°C and 5 minutes at 95°C.

Real time detection was performed on the ABI PRISM 7700 (PE Applied biosystems) using the Taqman protocol. This protocol includes an initiation of 10 min at 95°C, followed by 40 cycles (15 seconds at 95°C, and 1 minute at 60°C). mRNA levels of 18S were used as an internal control. Each sample was analyzed in duplicate. Relative gene expressions were calculated using the ΔΔCt method. The primers (Invitrogen) and probes (Eurogentec) used, are described in **table 1**.

Table 1 Primers and probes sequences used for real time qPCR

Gene		Primers
18S	Sense	5′-CGG CTA CCA CAT CCA AGG A-3′
	Anti-sense	5′-CCA ATT ACA GGG CCT CGA AA-3′
	Probe	5′-CGC GCA AAT TAC CCA CTC CCG A -3′
αSMA	Sense	5′-GCC AGT CGC CAT CAG GAA C-3′
	Anti-sense	5′-CAC ACC AGA GCT GTG CTG TCT T-3′
	Probe	5′-CTT CAC ACA TAG CTG GAG CAG CTT CTC GA-3′
Collagen type 1	Sense	5′-TGG TGA ACG TGG TGT ACA AGG T-3′
	Anti-sense	5′-CAG TAT CAC CCT TGG CAC CAT-3′
	Probe	5′-TCC TGC TGG TCC CCG AGG AAA CA-3′
CYP1A2	Sense	5′-TCA CTG AAT GGC TTC CAC ATT C-3′
	Anti-sense	5′-CTG GGC GGA ACA CAA AGG-3′
	Probe	5′-CTT TCC ACT GCT TCT CAT CAT GGT TGA CCT-3′
iNOS	Sense	5′-GTG CTA ATG CGG AAG GTC ATG-3′
	Anti-sense	5′-CGA CTT TCC TGT CTC AGT AGC AAA-3′
	Probe	5′-CCC GCG TCA GAG CCA CAG TCC T-3′
TGFβ	Sense	5′-GGG CTA CCA TGC CAA CTT CTG-3′
	Anti-sense	5′-GAG GGC AAG GAC CTT GCT GTA-3′
	Probe	5′-CCT GCC CCT ACA TTT GGA GCC TGG A-3′
TNFα	Sense	5′-GTA GCC CAC GTC GTA GCA AAC-3′
	Anti-sense	5′-AGT TGG TTG TCT TTG AGA TCC ATG-3′
	Probe	5′-CGCTGGCTCAGCCACTCCAGC-3′

Western blot

Western blot analysis of total cell lysates was performed by SDS-PAGE followed by semi dry-blotting to transfer the proteins to Hybond ECL nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). Ponceau S 0.1 % w/v (Sigma-Aldrich, St. Louis, MO, USA) staining was used to confirm electrophoretic transfer and was used as a loading control. Protein expression of α-SMA was detected using an antibody against α-SMA (Sigma-Aldrich, St. Louis, MO, USA, mouse monoclonal, dilution 1:2000). The blots were analyzed in a ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA). Protein band intensities were quantified by Image lab software (Bio-Rad, Hercules, CA, USA).

Immunofluorescence

Activated HSCs were cultured on glass coverslips. After treatment, coverslips were fixed in 4% paraformaldehyde/PBS. Prior to staining, cells were permeabilized in 1% Triton-X100 for 5 minutes. Nonspecific antibody binding sites were blocked for 30 min in 0.5% BSA/PBS. Then, cells were incubated with primary antibodies (α-SMA: Sigma-Aldrich, St. Louis, MO, USA, dilution 1:500; collagen 1α1: Southern Biotech, dilution

1:30) for 1-2 hours. As secondary antibodies we used the Alexa fluorophores (Molecular Probes, diluted 1:500). Finally, coverslips were mounted in fluorescence mounting medium S3023 containing DAPI for nuclear staining (DAKO). The slides were visualized using the confocal laser scanning microscope (Zeiss 410 inverted laser scan microscope).

BrdU incorporation ELISA assay

Proliferation was measured using a BrdU incorporation ELISA assay (Roche Diagnostics, Almere, the Netherlands) according to the manufacturer's instructions.

Real time proliferation assay

Real time proliferation of the myofibroblasts was assessed using an xCELLigence Real-time Cell Analyzer DP system (ACAE Biosciences, San Diego, USA), a system using electrical impedance as a measurement of both proliferation and stretching. When the cell number and/or size increase, impedance will increase. Per well 5000 cells were plated, after an attachment period of 4 hours cells were incubated with the indicated compounds.

Statistical analysis

All results are presented as the mean of at least three independent experiments using cells from different rats ± standard deviation. Statistical analysis of the results was performed using GraphPad Prism 5 for Windows. A student t-test or two-way ANOVA followed by Bonferroni's multiple comparison tests were used to determine significant differences between the groups. A p-value <0.05 was considered statistically significant.

Results

Caffeine reduces α-SMA and collagen-1α1 mRNA as well as protein expression in hepatic stellate cells and in portal myofibroblasts

Upon treatment with caffeine for 72h both hepatic stellate cells and portal myofibroblasts showed reduced mRNA expression of the fibrogenic markers α-SMA and collagen type 1 mRNA **(Figure 1)**. Also at the protein level, α-SMA and collagen type 1 expression were reduced upon treatment with caffeine. Using Western blot and immunofluorescence staining, we were able to show a decrease of α-SMA compared to untreated cells **(Figure 2A-B)**. Collagen type 1 expression disappeared almost completely upon caffeine treatment as assessed by immunofluorescence staining **(Figure 2C)**.

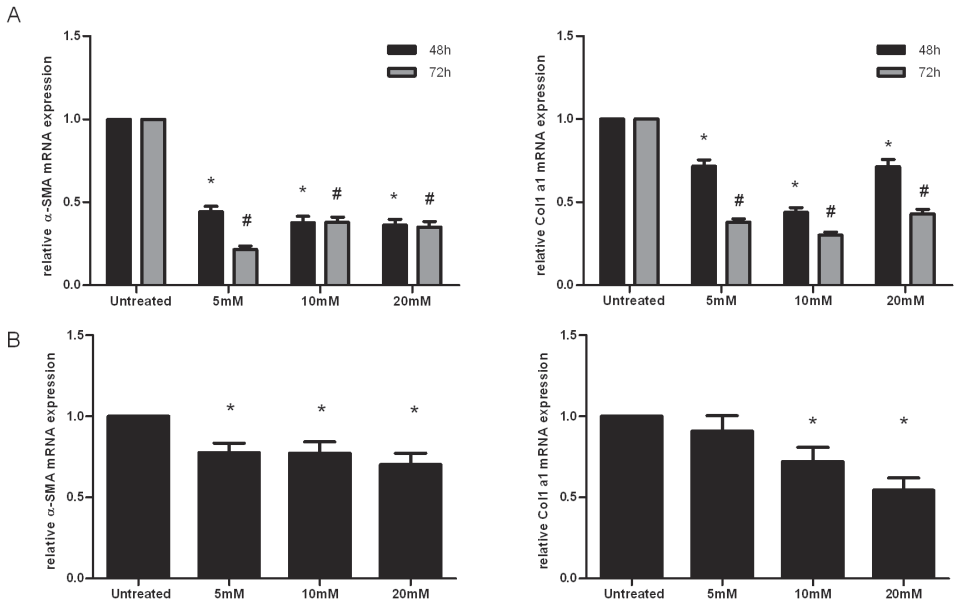


Figure 1. Caffeine reduces α-SMA as well as collagen mRNA levels in hepatic stellate cells as well as in portal myofibroblasts
A Hepatic stellate cells show reduced α-SMA and collagen 1α1 mRNA levels after 48h and 72h treatment with caffeine. * p<0.05 compared to untreated 48h; # p<0.05 compared to untreated 72h. **B** Portal myofibroblasts show reduced α-SMA and collagen 1α1 mRNA levels after 72h treatment with caffeine. * p<0.05 compared to untreated.

Caffeine reduces proliferation of hepatic stellate cells and portal myofibroblasts

In addition to markers of fibrogenesis, we investigated the effect of caffeine on the proliferation of stellate cells. Treatment of caffeine decreased proliferation of already activated stellate cells as shown by decreased BrdU incorporation. In addition, caffeine also prevented the proliferation of quiescent stellate cells during culture-activation **(Figure 3A)**. The proliferation during the culture-activation process was visualized using the xCELLigence, and we observed a dose-dependent decrease of the proliferation rate upon treatment with caffeine. Upon halting caffeine treatment, hepatic stellate cells started to proliferate again, indicating that they did not die due to the caffeine treatment **(Figure 3B)**.

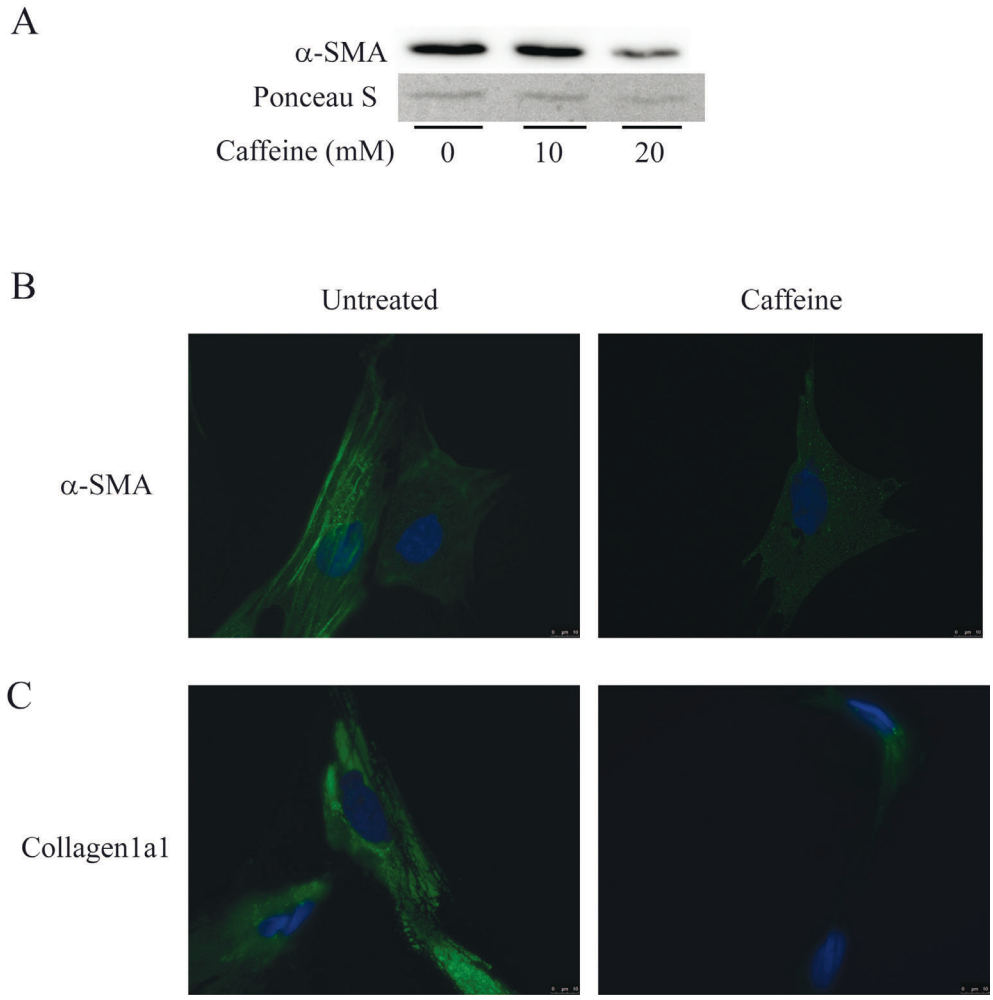


Figure 2. Caffeine reduces α-SMA as well as collagen protein levels in hepatic stellate cells
A Reduced α-SMA expression in hepatic stellate cells treated for 72h with indicated concentrations of caffeine. A representative blot of n=3 is shown. Loading control: Ponceau S staining.
B Immunofluorescent staining shows a decreased expression of α-SMA in hepatic stellate cells treated for 72h with 20mmol/L of caffeine. α-SMA staining in green, nuclear DAPI staining in blue. Magnification 630x.
C Immunofluorescent staining shows a decreased expression of collagen type 1 in hepatic stellate cells treated for 72h with 20mmol/L of caffeine. In green collagen type 1 staining, in blue nuclear DAPI staining. Magnification 630x.

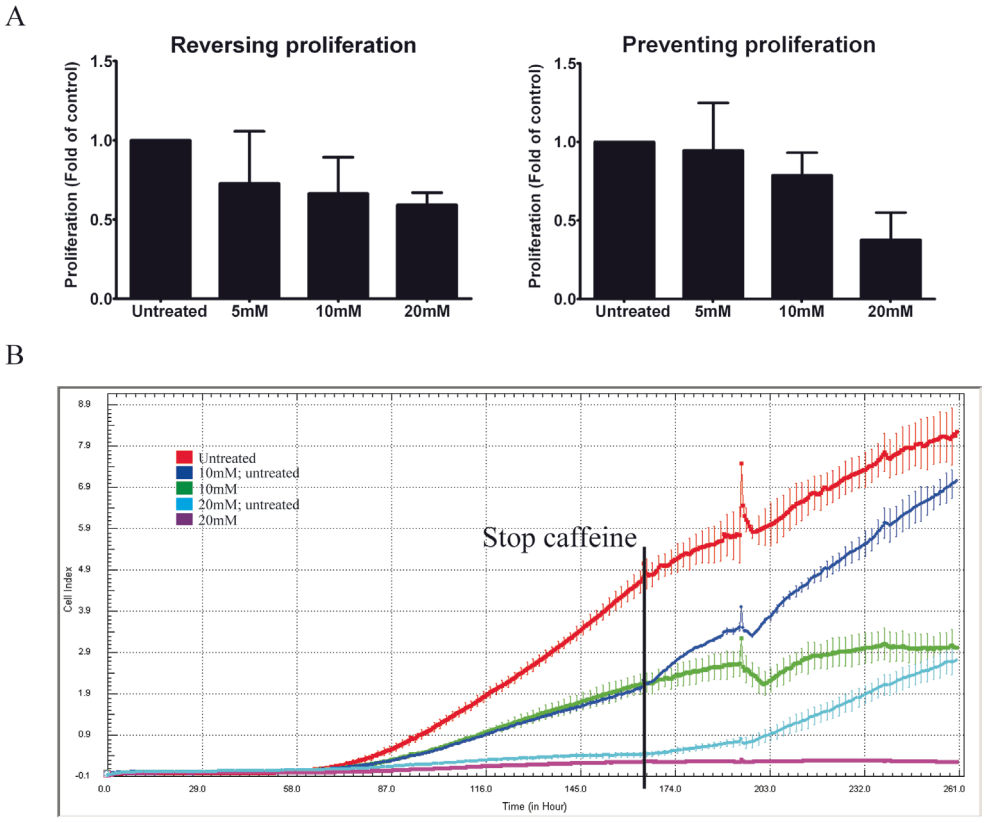


Figure 3. Proliferation of hepatic stellate cells is reduced upon treatment of caffeine
A In the left panel activated HSCs were incubated with different concentrations of caffeine for 72h. In the right panel, quiescent HSCs were incubated with different concentrations of caffeine during the first 5 days after isolation. Proliferation was assessed using a BrdU incorporation ELISA. Representative graphs of n=3 are shown.
B Real time monitoring of proliferation and growth of quiescent stellate cells incubated with caffeine undergoing culture-activation. Caffeine treatment can dose-dependently inhibit proliferation compared to untreated cells, but upon halting caffeine treatment the stellate cells are able to continue proliferating. Representative graph of n=3 is shown.

The metabolite of caffeine, 1,7-dimethylxanthine is also able to reduce α-SMA and collagen type 1 expression

Since it was shown previously that caffeine exerts its antifibrotic effect via its main metabolite, 1,7-dimethylxanthine (DMX) in hepatocytes and hepatic stellate cells, we determined whether this effect could be reproduced. The effects of 1,7-DMX on α-SMA and collagen type 1 expression were similar to the effects observed with caffeine. Moreover, the combination of caffeine and 1,7-DMX further enhanced the reduction of α-SMA and collagen type 1 expression. **(Figure 4)**

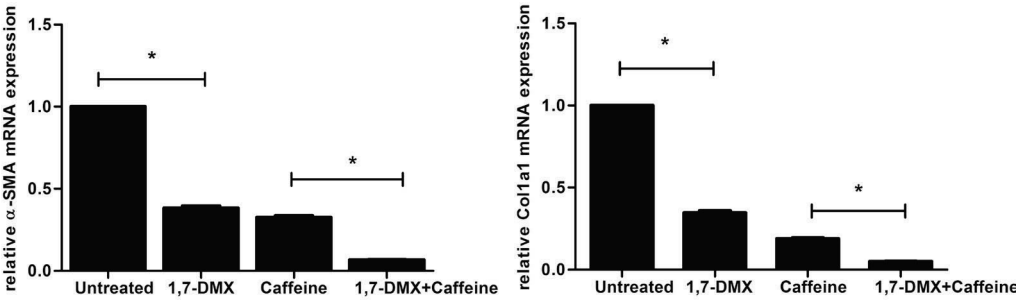


Figure 4. 1,7-DMX and caffeine have complementary effects reducing activation markers of activated hepatic stellate cells
Treatment of activated hepatic stellate cells during 72h with either 1 mmol/L 1,7-DMX or 20 mmol/L caffeine reduce α-SMA as well as collagen 1α1 mRNA expression compared to untreated cells. The combination of caffeine and 1,7-DMX reduces α-SMA and collagen 1α1 mRNA expression even further compared to caffeine alone. *p<0.05.

Mechanism of action

Caffeine is metabolized in the liver by hepatocytes through the enzyme cytochrome P450 1A2 (CYP1A2). To study whether our observed effects in stellate cells were caused by direct effects of caffeine or by its metabolite, we determined the expression of this enzyme in hepatic stellate cells and portal myofibroblasts. Compared to primary hepatocytes, both hepatic stellate cells and portal myofibroblasts hardly express CYP1A2 (>1000 fold less than hepatocytes), making the possibility of caffeine metabolism in HSCs and PMFs highly unlikely **(Figure 5)**.

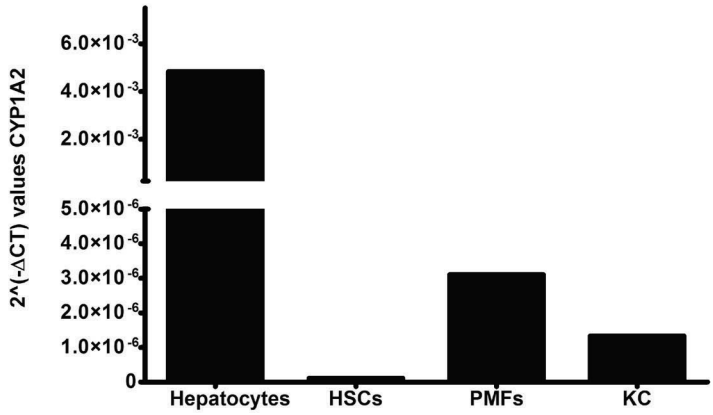


Figure 5. Activated hepatic stellate cells as well as portal myofibroblasts hardly express CYP1a2
Compared to hepatocytes there is hardly any expression of CYP1A2 mRNA in HSCs, PMFs and KCs (1000-fold difference). Results are shown in $2^{-\Delta CT}$ values, where Δ refers to the correction of cDNA using 18S values.

Caffeine and 1,7-DMX have anti-inflammatory properties

Inflammation plays an important role in fibrogenesis. Therefore, we also investigated the effect of caffeine and its main metabolite on Kupffer cells and hepatocytes. Kupffer cells pretreated with caffeine or 1,7-DMX showed reduced induction of LPS-induced TNF-α mRNA expression, with a more pronounced effect of caffeine **(Figure 6A)**.

When hepatocytes were pre-incubated with 1,7-DMX before exposure to an inflammatory cytokine mixture composed of TNF-α, IL-1β and IFN-γ, the expression of cytokine mixture-induced iNOS mRNA was attenuated **(Figure 6B)**.

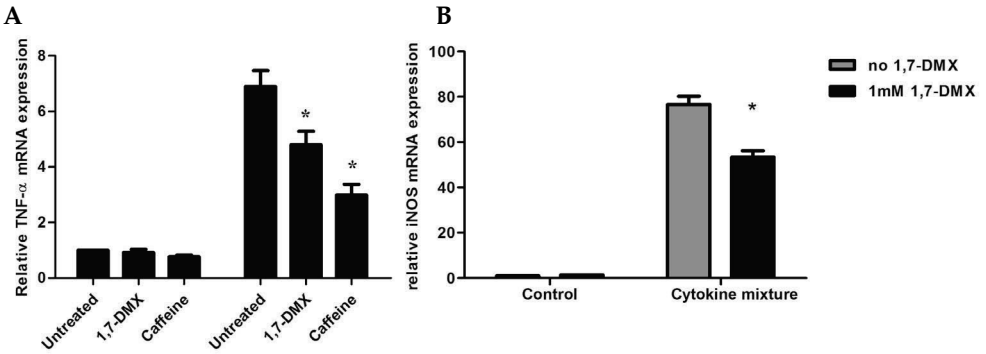


Figure 6. 1,7-DMX and caffeine show anti-inflammatory effects
A Kupffer cells pretreated with 1,7-DMX (1mmol/L) or caffeine (20mmol/L) for 30 minutes have reduced LPS-induced TNF-α mRNA levels compared to LPS (0.1μg/mL) treatment alone. *p<0.05 compared to LPS-treated cells.
B Pretreatment of primary hepatocytes with 1,7-DMX (1mmol/L) reduces cytokine mixture-induced iNOS mRNA expression levels compared to cytokine mixture treatment alone. *p<0.05 compared to cytokine mixture-treated cells.

Discussion

In this study, we demonstrate that caffeine has both a direct as well as an indirect antifibrotic effect. This is in line with literature, where effects of both caffeine and 1,7-dimethylxanthine have been described. The novelty of our data is that these effects are complementary and mediated via different pathways. Moreover, we demonstrate that caffeine has effects on additional cell types involved in liver fibrogenesis, such as Kupffer cells and hepatocytes.

Caffeine can have multiple effects, of which the most known is its function as a pan-adenosine receptor antagonist. Therefore, it is likely that the reduction of stellate cell activation and proliferation is mediated by inhibition of adenosine receptors. This

is confirmed in earlier studies, where it was shown that adenosine occupancy of the adenosine A2a receptor, the adenosine receptor present on HSCs, stimulates collagen production.(13) Also in dermal fibrosis, adenosine was implicated as a main contributor of extracellular matrix production.(14)

In our study, the effects of 1,7-DMX and caffeine treatment on hepatic stellate cells are similar. Since there is no CYP1A2 expression in hepatic stellate cells to metabolize caffeine, there must be an indirect effect of caffeine as well. When caffeine is metabolized by hepatocytes, via the enzyme CYP2A1, three metabolites are produced; 1,7-DMX (or paraxanthine), theobromine and theophylline. 1,7-DMX is the main metabolite, representing 80% out of all the metabolites. It has been shown before that 1,7-DMX is able to reduce fibrogenic factors, like transforming growth factor-β (TGF-β) and connective tissue growth factor (CTGF), although the exact mechanism remains to be clarified.

A well-known derivative of theobromine is pentoxifylline, a phosphodiesterase inhibitor known to inhibit tumor necrosis factor–α (TNF-α) synthesis by increasing intracellular cAMP concentrations.(15) Previously, it was shown that pentoxifylline is able to decrease proliferation (16) and collagen production (17) of hepatic stellate cells. Randomized controlled trials have also shown a beneficial effect of pentoxifylline treatment in liver disease, for example complications in advanced liver cirrhosis were reduced (18) and short-term survival in patients with severe alcoholic hepatitis was improved (19).

It is known that caffeine antagonizes adenosine receptors at low concentrations (20). The concentrations we used, up to 20 mmol/L, are at the high end of the range. At high concentrations caffeine is known to act as a phosphodiesterase inhibitor. As described above, this could result in anti-inflammatory effects, due to increased intracellular cyclic adenosine monophosphate (cAMP) concentrations, which inhibits TNF-α synthesis.(21) We observe similar anti-inflammatory effects in Kupffer cells and hepatocytes. However, whether these anti-inflammatory effects are beneficial in chronic liver disease remains to be elucidated, since it has been described that caffeine may also exacerbate liver inflammation by inhibiting adenosine receptors, which are critical in immune cells in the production of pro-inflammatory cytokines needed to exert physiological effects.(22)

Numerous reports suggest coffee as an anti-oxidant (23-26), however, whether this is due to caffeine and its metabolites is unclear. Next to caffeine, there are many other, less well-studied, ingredients in coffee that could also be responsible for the anti-

oxidative properties of coffee. For example, the diterpenes cafestol and kahweol, absent in filtered coffee, have been shown to induce glutathione and HO-1 levels through Nrf2 (nuclear factor erythroid 2-related factor)-mediated gene regulation, thereby protecting against oxidative damage. (27) However, there is also evidence that these ingredients are responsible for raising LDL (low-density lipoprotein) cholesterol.(28) Other important ingredients of coffee are chlorogenic acids. These are presumed to be major antioxidants, by inhibiting reactive oxygen species, as well as scavenging them.(29) Also anti-inflammatory effects of chlorogenic acids have been described, for example inhibition of LPS-induced cyclooxygenase-2 (COX-2) expression in macrophages, resulting in reduced prostaglandin E2 expression.(30) However, negative effects have been described as well, such as increased homocysteine levels, associated with atherosclerosis.(31) To determine the effect of coffee, future experiments should include incubation of liver myofibroblasts with a raw extract of coffee as well as the ingredients described above.

To summarize, from this study we conclude that caffeine exerts both a direct as well as an indirect antifibrotic effect *in vitro*. Moreover, we confirmed its anti-inflammatory action in our *in vitro* model of Kupffer cells and hepatocytes stimulated with cytokine mixture. This study provides further evidence for protective actions of caffeine, and possibly coffee, in patients with liver disease.

Acknowledgements

Microscopic fluorescence imaging was performed at the UMCG Microscopy & Imaging Center (UMIC), which is supported by the Netherlands Organisation for Health Research and Development (ZonMW grant 40-00506-98-9021).

References

(1) Molloy JW, Calcagno CJ, Williams CD, Jones FJ, Torres DM, Harrison SA. Association of coffee and caffeine consumption with fatty liver disease, nonalcoholic steatohepatitis, and degree of hepatic fibrosis. Hepatology 2012 Feb;55(2):429-436.

(2) Freedman ND, Everhart JE, Lindsay KL, Ghany MG, Curto TM, Shiffman ML, et al. Coffee intake is associated with lower rates of liver disease progression in chronic hepatitis C. Hepatology 2009 Nov;50(5):1360-1369.

(3) Modi AA, Feld JJ, Park Y, Kleiner DE, Everhart JE, Liang TJ, et al. Increased caffeine consumption is associated with reduced hepatic fibrosis. Hepatology 2010 Jan;51(1):201-209.

(4) Birerdinc A, Stepanova M, Pawloski L, Younossi ZM. Caffeine is protective in patients with non-alcoholic fatty liver disease. Aliment Pharmacol Ther 2012 Jan;35(1):76-82.

(5) Moreno MG, Chavez E, Aldaba-Muruato LR, Segovia J, Vergara P, Tsutsumi V, et al. Coffee prevents CCl(4)-induced liver cirrhosis in the rat. Hepatol Int 2011 Sep;5(3):857-863.

(6) Furtado KS, Prado MG, Aguiar E Silva MA, Dias MC, Rivelli DP, Rodrigues MA, et al. Coffee and caffeine protect against liver injury induced by thioacetamide in male Wistar rats. Basic Clin Pharmacol Toxicol 2012 Nov;111(5):339-347.

(7) Lv X, Chen Z, Li J, Zhang L, Liu H, Huang C, et al. Caffeine protects against alcoholic liver injury by attenuating inflammatory response and oxidative stress. Inflamm Res 2010 Aug;59(8):635-645.

(8) Klemmer I, Yagi S, Gressner OA. Oral application of 1,7-dimethylxanthine (paraxanthine) attenuates the formation of experimental cholestatic liver fibrosis. Hepatol Res 2011 Nov;41(11):1094-1109.

(9) Moshage H, Casini A, Lieber CS. Acetaldehyde selectively stimulates collagen production in cultured rat liver fat-storing cells but not in hepatocytes. Hepatology 1990 Sep;12(3 Pt 1):511-518.

(10) Conde de la Rosa L, Schoemaker MH, Vrenken TE, Buist-Homan M, Havinga R, Jansen PL, et al. Superoxide anions and hydrogen peroxide induce hepatocyte death by different mechanisms: involvement of JNK and ERK MAP kinases. J Hepatol 2006 May;44(5):918-929.

(11) Kinnman N, Francoz C, Barbu V, Wendum D, Rey C, Hultcrantz R, et al. The myofibroblastic conversion of peribiliary fibrogenic cells distinct from hepatic stellate cells is stimulated by platelet-derived growth factor during liver fibrogenesis. Lab Invest 2003 Feb;83(2):163-173.

(12) Teufelhofer O, Parzefall W, Kainzbauer E, Ferk F, Freiler C, Knasmuller S, et al. Superoxide generation from Kupffer cells contributes to hepatocarcinogenesis: studies on NADPH oxidase knockout mice. Carcinogenesis 2005 Feb;26(2):319-329.

(13) Chan ES, Montesinos MC, Fernandez P, Desai A, Delano DL, Yee H, et al. Adenosine A(2A) receptors play a role in the pathogenesis of hepatic cirrhosis. Br J Pharmacol 2006 Aug;148(8):1144-1155.

(14) Chan ES, Liu H, Fernandez P, Luna A, Perez-Aso M, Bujor AM, et al. Adenosine A2A receptors promote collagen production by a Fli1- and CTGF-mediated mechanism. Arthritis Res Ther 2013 May 11;15(3):R58.

(15) Windmeier C, Gressner AM. Pharmacological aspects of pentoxifylline with emphasis on its inhibitory actions on hepatic fibrogenesis. Gen Pharmacol 1997 Aug;29(2):181-196.

(16) Kawada N, Uoya M, Seki S, Kuroki T, Kobayashi K. Regulation by cAMP of STAT1 activation in hepatic stellate cells. Biochem Biophys Res Commun 1997 Apr 17;233(2):464-469.

(17) Romanelli RG, Caligiuri A, Carloni V, DeFranco R, Montalto P, Ceni E, et al. Effect of pentoxifylline on the degradation of procollagen type I produced by human hepatic stellate cells in response to transforming growth factor-beta 1. Br J Pharmacol 1997 Nov;122(6):1047-1054.

(18) Lebrec D, Thabut D, Oberti F, Perarnau JM, Condat B, Barraud H, et al. Pentoxifylline does not decrease short-term mortality but does reduce complications in patients with advanced cirrhosis. Gastroenterology 2010 May;138(5):1755-1762.

(19) Akriviadis E, Botla R, Briggs W, Han S, Reynolds T, Shakil O. Pentoxifylline improves short-term survival in severe acute alcoholic hepatitis: a double-blind, placebo-controlled trial. Gastroenterology 2000 Dec;119(6):1637-1648.

(20) Fredholm BB, Battig K, Holmen J, Nehlig A, Zvartau EE. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacol Rev 1999 Mar;51(1):83-133.

(21) Horrigan LA, Kelly JP, Connor TJ. Immunomodulatory effects of caffeine: friend or foe? Pharmacol Ther 2006 Sep;111(3):877-892.

(22) Ohta A, Lukashev D, Jackson EK, Fredholm BB, Sitkovsky M. 1,3,7-Trimethylxanthine (Caffeine) may Exacerbate Acute Inflammatory Liver Injury by Weakening the Physiological Immunosuppressive Mechanism. J Immunol 2007 Dec 1;179(11):7431-7438.

(23) Yazheng L, Kitts DD. Activation of antioxidant response element (ARE)-dependent genes by roasted coffee extracts. Food Funct 2012 Sep;3(9):950-954.

(24) Gutierrez-Grobe Y, Chavez-Tapia N, Sanchez-Valle V, Gavilanes-Espinar JG, Ponciano-Rodriguez G, Uribe M, et al. High coffee intake is associated with lower grade nonalcoholic fatty liver disease: the role of peripheral antioxidant activity. Ann Hepatol 2012 May-Jun;11(3):350-355.

(25) Gomez-Ruiz JA, Leake DS, Ames JM. In vitro antioxidant activity of coffee compounds and their metabolites. J Agric Food Chem 2007 Aug 22;55(17):6962-6969.

(26) Niseteo T, Komes D, Belscak-Cvitanovic A, Horzic D, Budec M. Bioactive composition and antioxidant potential of different commonly consumed coffee brews affected by their preparation technique and milk addition. Food Chem 2012 Oct 15;134(4):1870-1877.

(27) Cavin C, Holzhaeuser D, Scharf G, Constable A, Huber WW, Schilter B. Cafestol and kahweol, two coffee specific diterpenes with anticarcinogenic activity. Food Chem Toxicol 2002 Aug;40(8):1155-1163.

(28) Weusten-Van der Wouw MP, Katan MB, Viani R, Huggett AC, Liardon R, Liardon R, et al. Identity of the cholesterol-raising factor from boiled coffee and its effects on liver function enzymes. J Lipid Res 1994 Apr;35:721-733.

(29) Upadhyay R, Mohan Rao LJ. An outlook on chlorogenic acids-occurrence, chemistry, technology, and biological activities. Crit Rev Food Sci Nutr 2013;53(9):968-984.

(30) Shan J, Fu J, Zhao Z, Kong X, Huang H, Luo L, et al. Chlorogenic acid inhibits lipopolysaccharide-induced cyclooxygenase-2 expression in RAW264.7 cells through suppressing NF-kappaB and JNK/AP-1 activation. Int Immunopharmacol 2009 Aug;9(9):1042-1048.

(31) Olthof MR, Hollman PC, Zock PL, Katan MB. Consumption of high doses of chlorogenic acid, present in coffee, or of black tea increases plasma total homocysteine concentrations in humans. Am J Clin Nutr 2001 Mar;73(3):532-538.

Chapter 6

Summary, general discussion and future perspectives

MH Tiebosch, KN Faber, H Moshage

Chronic liver disease is a major health burden, often resulting in fibrosis or its end stage cirrhosis, irrespective of the etiology. A chronic wound healing response occurs upon persisting injury, which causes hepatocytes to die. However, myofibroblasts, responsible for the deposition of extracellular matrix, flourish. Possibly, adaptive mechanisms of myofibroblasts (mainly originating from stellate cells) can explain this paradox. One of these mechanisms could be an altered anti-oxidant status, as oxidative stress plays a major role in fibrogenesis.

In this thesis, we aimed to investigate the mechanism(s) of hepatic stellate cell activation focusing on the role of oxidative stress and anti-oxidant mechanisms, in order to contribute to a stellate cell-targeted therapy for liver fibrosis.

In **chapter 2**, we investigated the role of heme oxygenase-1 (HO-1) in cells most intimately involved in liver fibrogenesis: hepatic stellate cells and macrophages. HO-1 is an enzyme heavily involved in oxidative stress. Previously, we have shown that HO-1 induction can protect hepatocytes from superoxide-induced cell death.(1) Since there are also reports that HO-1 is antifibrotic and immunomodulatory, we investigated the role of HO-1 in *in vitro* cultures of hepatic stellate cells and macrophages.

The main finding of this study was that HO-1 appears to be antifibrotic due to its immunomodulatory effects, since we showed that HO-1 induction in macrophages changes the phenotype of the macrophages to the pro-inflammatory M1 phenotype, while markers of the tissue remodeling and profibrotic M2 macrophage phenotype are decreased. This suggests that HO-1 is able to regulate the actions of macrophages, which can then orchestrate other cells, for example myofibroblasts. To unequivocally demonstrate that HO-1 is antifibrotic via immunomodulatory effects, a co-culture system of hepatic stellate cells and Kupffer cells in which HO-1 expression is induced should be established. Alternatively, an *in vivo* model of liver fibrosis, with specific induction (or knockout/down) of HO-1 in Kupffer cells, could be developed.

The fact that the phenotype of the macrophages is changed to the pro-inflammatory M1 phenotype appears to be an undesired response at first glance. However, we hypothesize that an initial induction of inflammation is favourable over a profibrotic response, because it will accelerate the resolution and/or clearance of the causative agent of the inflammatory reaction, thereby limiting long-term activation of stellate cells and fibrosis. This is supported by recent reports that show an increased phagocytosis and bacterial clearance of macrophages upon HO-1 induction.(2-4) In order to further elucidate these mechanisms, the macrophage phenotype should be investigated in an

in vivo liver disease model, with or without induction of HO-1 or in conditional HO-1 knockout mice.

The origin of macrophages involved in fibrogenesis has to be studied in more detail, as different subsets of macrophages exist in liver disease.(5) The increase of ED-1 expression we observed in our study could originate from an increase of extrahepatic macrophages migrating to the liver from, for example, the bone marrow. It will be of interest to know whether these extrahepatic macrophages behave differently compared to resident Kupffer cells. A recent study showed that dendritic cells do not play a role in liver fibrosis.(6)

Taking into account that our previous study showed a protective effect of HO-1 in hepatocytes against superoxide anion-induced cell death, HO-1 emerges as a central beneficial enzyme. The mechanisms of all these protective actions are not yet fully elucidated. HO-1 is responsible for the enzymatic conversion of heme into carbon monoxide, free iron and biliverdin. We previously showed that HO-1 protects hepatocytes via carbon monoxide (CO).(1) It is likely that in other cell types the protective mechanism of HO-1 is similar to that of hepatocytes. To study this, Kupffer cells should be cultured in a CO-containing atmosphere, or incubated with CO-releasing molecules (CORMs). There is evidence that CO is also responsible for HO-1-mediated actions in macrophages. (2-4) Another suggested mechanism is protection by biliverdin, to which antioxidative properties are attributed.(7)

In chapter 2, we focused on extracellular oxidative stress as part of an inflammatory response, which therefore is a main driving force for fibrosis. Intracellular oxidative stress is believed to play a role in the activation of hepatic stellate cells as well, and since the liver paradox can possibly be explained by different adaptive mechanisms to this intracellular oxidative stress, we focused on these mechanisms in chapter 3 and 4.

In **chapter 3**, we investigated the role of antioxidant systems in the resistance of activated hepatic stellate cells against ROS-induced toxicity, in particular hydrogen peroxide and superoxide anions. Hydrogen peroxide can be processed by catalase and glutathione peroxidase into water and oxygen. In this study, we demonstrate that upon activation of stellate cells intracellular glutathione content and glutathione peroxidase are increased, accompanied by a dramatic decrease of catalase and mitochondrial manganese superoxide dismutase (MnSOD) protein levels.

Expression of Glutathione peroxidase 1 (GPx1) is increased upon activation, resulting in an enhanced resistance against oxidative stress. GPx1 is known to convert reduced

glutathione into oxidized glutathione, thereby neutralizing hydrogen peroxide. The fact that glutathione is essential for the activity of GPx1 can explain the complementary increase of glutathione upon HSC activation. Previous studies have shown that GPx1 overexpression leads to a protection against oxidative stress-induced damage.(8-10) On the other hand, catalase protein expression drops dramatically upon HSC activation. Possibly, the increase of glutathione and GPx1 during the activation of HSCs is more important than the decrease of catalase in the detoxification of hydrogen peroxide. Blocking catalase and GPx activity separately or in combination resulted in sensitization of stellate cells to apoptosis, which was further increased upon exposure to hydrogen peroxide. Apparently, both enzymes play a key protective role against apoptosis in activated hepatic stellate cells even though catalase levels are very low.

Depletion of glutathione resulted in increased hydrogen peroxide-induced necrosis, which contrasts to the caspase-3-dependent apoptosis upon inhibiting catalase and GPx1 activity. This could be explained by a changed redox state upon the combination of glutathione depletion and exposure to reactive oxygen species, as glutathione is the most important regulator of the redox state.(11,12) Therefore, glutathione is indispensable for apoptosis as a change in the redox state probably affects the activity of caspases, because this requires reduced sulfhydryl groups.(13)

The study described in Chapter 3 demonstrates an adaptive mechanism of stellate cells acquired during their activation process. The increase in glutathione makes stellate cells more resistant to necrotic cell death, while preserving the potential to undergo apoptotic cell death. This could explain the feature of apoptotic clearance of hepatic stellate cells during the resolution of fibrosis.

The decrease in MnSOD expression does not appear to fit in this hypothesis of adaptation to a hostile pro-oxidant environment. Reactive oxygen species in liver fibrogenesis consist of hydrogen peroxide, but also superoxide and hydroxyl radicals. Apparently, HSC are adapted to deal with hydrogen peroxide, however, their defense against superoxides appears weak with reduced MnSOD en unchanged CuZnSOD expression.

Therefore in **chapter 4**, we further studied the role of MnSOD in hepatic stellate cells and we investigated the feasibility of reversing the phenotype of activated hepatic stellate cells to a quiescent phenotype by inducing MnSOD levels. The main finding of this study was that artificial overexpression of MnSOD led to decreased expression of activation markers in hepatic stellate cells.

Previously, it was shown that in two different *in vivo* models of liver disease, cholestatic (14) and alcoholic liver disease (15), adenoviral-mediated overexpression of MnSOD attenuated liver injury. Hepatocyte death was inhibited, as shown by a reduction of biochemical markers, and fibrosis was reduced as well. Moreover, lipid peroxidation as well as the inflammatory response was reduced. The protective effects described in these studies are most likely due to direct effects of MnSOD induction in the hepatocytes. With induced MnSOD levels, these cells are better equipped to handle damaging stimuli and therefore the inflammatory response, and as a result fibrosis, will be reduced. In our study, however, we have shown that MnSOD induction in hepatic stellate cells can also protect against liver fibrosis. Thus, MnSOD can play a general protective role in liver disease.

The decrease of MnSOD levels upon HSC activation was a surprising finding, which appears to contradict our hypothesis that stellate cells have an increased resistance to reactive oxygen species, as MnSOD is required to detoxify superoxide anions produced in the mitochondria. However, there are several hypotheses to explain this apparent contradiction and they will be discussed in the following paragraphs.

Recently, it was shown that stellate cells switch their metabolism during activation from oxidative phosphorylation to aerobic glycolysis.(16) During this metabolic shift the number of mitochondria is retained, and even increased upon activation when comparing activated HSC to freshly isolated quiescent HSC. This is known as the Warburg effect, which is commonly observed in cancer cells. Interestingly, MnSOD expression is also reduced in cancer cells.(17)

It is suggested that this is due to an increased demand for mitochondrial ROS to stabilize hypoxia inducible factor-1α (HIF-1α) expression (18), which is required to promote the Warburg effect (19,20) and is therefore indispensable for creating a survival advantage. The survival advantage of glycolysis for cancer cells lies in a shortage of oxygen for aerobic oxidative phosphorylation in the hypoxic microenvironment of a tumor.(21) In liver diseases, the microenvironment is also relatively hypoxic and it is believed that hypoxia contributes to the activation of hepatic stellate cells (22). Hepatic stellate cells are exposed to hypoxia during the isolation procedure and we hypothesize that MnSOD levels drop during activation as a result of this hypoxia. This would then lead to a shift in metabolism, which provides advantages for the myofibroblast phenotype of the stellate cell (for example proliferation). Induction of MnSOD levels could therefore change the metabolism back to oxidative phosphorylation, and thereby

change the activated phenotype of stellate cells to a quiescent phenotype. A strategy to test this hypothesis would be to increase the oxygen concentration during isolation and culture. According to our hypothesis, MnSOD expression should then not decrease and the hepatic stellate cells should not undergo culture-activation.

However, it has also been reported that the Warburg effect in cancer cells is caused by the changed requirements of the cell rather than hypoxia.(23) Although aerobic glycolysis appears to be inefficient as only four adenosine triphosphate (ATP) molecules are produced (instead of thirty six), all the energy and biomass that is not used to make ATP in the oxidative phosphorylation can be used for nucleotide and lipid production. If MnSOD would lead to a change in metabolism back to oxidative phosphorylation, proliferation would decrease/cease as there are not enough means to provide for constant mitosis.

Another effect of induction of MnSOD expression could be an increase in the production of hydrogen peroxide as more superoxides are converted into hydrogen peroxide. As we have shown in chapter 3, stellate cells have an increased resistance against hydrogen peroxide-induced cell death, due to an increase in glutathione and glutathione peroxidase 1 content. Therefore, we do not expect cell death regardless of high concentrations of hydrogen peroxide, especially since we did not observe increased cell death upon induction of MSOD levels. Moreover, hydrogen peroxide is described as a cell signaling molecule (24), and we previously observed a reduced proliferation of stellate cells upon hydrogen peroxide treatment (25). This is confirmed by other studies in which a redox control of the cell cycle has been described: a reduction of MnSOD results in increased superoxide generation and decreased hydrogen peroxide exposure, which is associated with entry into the S-phase of the cell cycle. On the other hand, an increase of MnSOD levels, and thereby a decrease of superoxide anion generation and increased exposure to hydrogen peroxide, results in cell cycle exit and quiescence/senescence of the cell.(26) This mechanism was established in embryonic myofibroblasts and it seems likely that this also holds true for hepatic stellate cells.

Caffeine appears to have antifibrotic properties in epidemiological human (27-30) and *in vivo* animal studies (31-34). Therefore, in **chapter 5** we investigated the effect of caffeine *in vitro* on hepatic stellate cells and macrophages. The main finding of this study was that both caffeine and its main metabolite 1,7-dimethylxanthine (1,7-DMX) have an antifibrotic effect *in vitro.* These effects are additive, but probably mediated via different pathways, since we showed that both HSCs and PMFs have hardly any expression of the caffeine-metabolizing enzyme CYP1A2.

The observed additive antifibrotic effect of 1,7-DMX and caffeine in hepatic stellate cells and portal myofibroblasts can be explained by a dual effect. On the one hand 1,7-DMX can act directly on myofibroblasts by inhibiting connective tissue growth factor (CTGF) and TGF-β expression as described earlier.(33,35) On the other hand caffeine can act directly on myofibroblasts via different mechanisms as will be explained in the next paragraphs.

Caffeine is known for its capacity to act as a pan-adenosine receptor antagonist (36), and these receptors are known to play a role in fibrosis (37). HSC express the adenosine 2a (A2a) receptor and it was previously shown that activation of this receptor causes activation of the stellate cells leading to increased collagen production.(38,39) Specific inhibition of this adenosine receptor in hepatic stellate cells could demonstrate if caffeine exerts its effects via adenosine receptor inhibition. However, it is known that only at low concentrations caffeine acts as a pan-adenosine receptor antagonist.(36) Since fairly high concentrations were used in our studies, it is unlikely that adenosine receptor antagonism is responsible for the observed effects of caffeine in hepatic stellate cells.

At high concentrations, caffeine can act as a phosphodiesterase (PDE) inhibitor (36), a pathway known to result in anti-inflammatory effects via the inhibition of TNF-α synthesis (40,41). Moreover, previous studies showed that the PDE inhibitor pentoxifylline, a derivative of theobromine (another metabolite of caffeine), is antifibrotic both *in vitro* in HSCs and also *in vivo*.(42-45) This could also be an explanation for the observed antifibrotic effect of caffeine in our study. Pentoxifylline is already used in clinical treatment of alcoholic liver disease, in order to prevent the hepatorenal syndrome.(46)

In addition we show an anti-inflammatory effect of 1,7-DMX pretreatment in hepatocytes and 1,7-DMX and caffeine pretreatment in LPS-stimulated Kupffer cells. This could result in an indirect antifibrotic effect by reducing the inflammatory response involved in fibrogenesis. Therefore, caffeine may protect against liver fibrosis by acting on multiple liver cell types.

Other ingredients of coffee (other than caffeine) are believed to have antioxidant properties, for example diterpenes and chlorogenic acids. Diterpenes are suggested to induce glutathione and HO-1 levels, protecting against oxidative damage.(47) In chapter 2, we showed that an induction of HO-1 did not have a direct antifibrotic effect on stellate cells, but rather an indirect effect via modulation of the inflammatory response. Increased glutathione content is beneficial for the redox status of the cell, and as we have shown in chapter 3, protects stellate cells against hydrogen peroxide-induced necrosis.

Therefore, we hypothesize that the antifibrotic effect of diterpenes in stellate cells is not very important, and that beneficial effects of diterpenes are especially due to anti-inflammatory mechanisms.

Chlorogenic acids are suggested to act as antioxidants via the ability to reduce reactive oxygen species production as well as scavenging radicals.(48) Moreover, they are also thought to have anti-inflammatory effects, via the inhibition of LPS-induced cyclooxygenase-2 (COX-2) expression in macrophages.(49) Therefore, it will be interesting to investigate the effect of chlorogenic acids on stellate cells directly, in order to find alterations in the anti-oxidant system, as well as to study their possible anti-inflammatory potential on Kupffer cells.

From bench to bedside? Clinical/translational perspectives

This thesis describes basic research on stellate cells, however, our final goal is to develop a stellate cell-targeted therapy for patients who suffer from liver fibrosis, or who are prone to develop fibrosis (for example patients with steatohepatitis). Therefore, in this final paragraph some possible translational mechanisms are described.

This thesis once again describes that fibrogenesis is a complicated process, with multiple factors playing a role. Instead of solely focusing on the reversal of the activated phenotype of stellate cells to a quiescent one, the hostile microenvironment should also be considered. Inflammation and macrophages in particular, play(s) a decisive role in maintaining a profibrogenic microenvironment. Therefore, the reversal of only HSC activation will not contribute to diminish fibrosis as long as the profibrogenic environment is maintained. On the other hand, therapies that focus only on reducing inflammation have shown to be ineffective as well. Therefore, development of a therapy aimed at both aspects should be pursued.

Treatment with heme oxygenase-1 is a good example of this. Although it does not have a direct antifibrotic effect on stellate cells, its *in vivo* protective properties could be explained by immunomodulatory effects. Therefore, future studies should focus on the direct effect of the altered phenotype of macrophages on the activation of hepatic stellate cells. As the protective effect of HO-1 seems to be a general phenomenon in liver disease, induction of HO-1 or (local) administration of carbon monoxide (the main protective product of HO-1) holds great promise for the treatment of chronic liver diseases.

Increased resistance of stellate cells against oxidative stress and cell death is crucial to consider when developing a therapy to eliminate activated stellate cells from the fibrotic liver. Furthermore, it is important to consider that stellate cells have increased resistance to necrosis, but can still be susceptible to apoptotic cell death. When targeting stellate cells with a therapy aimed to kill them, hepatocyte protection should be considered. Furthermore, the effect of dying hepatic stellate cells on the microenvironment, for example by provoking a new inflammatory response, should be considered as well.

As stellate cells resemble cancer cells in several aspects, i.e. their ability to proliferate, change metabolism etc., a similar therapeutic approach can be considered. Chemotherapeutic agents developed to specifically target cancer cell characteristics, for example their proliferation or glycolytic metabolism, can also be very useful to eliminate stellate cells. However, the major disadvantage of chemotherapeutic agents are the severe side effects, especially when these agents have to be administered to patients whose liver is damaged, but who are still unsure of actually developing liver cirrhosis.

The most obvious clinically relevant study of this thesis is the study on the effect of caffeine on several cell types involved in liver fibrosis. This project started with a clinical question: why is coffee or caffeine associated with a lower incidence of fibrosis? We show that caffeine can have protective effects via multiple mechanisms: directly via inhibition of proliferation and activation of myofibroblasts and indirectly via reduction of the inflammatory response. This study can contribute to the development of a caffeine-based therapy, in which the therapy is not only based on drinking coffee or other caffeine-containing beverages, but rather a specific targeted therapy where side effects can be minimized.

References

(**1**) Conde de la Rosa L, Vrenken TE, Hannivoort RA, Buist-Homan M, Havinga R, Slebos DJ, et al. Carbon monoxide blocks oxidative stress-induced hepatocyte apoptosis via inhibition of the p54 JNK isoform. Free Radic Biol Med 2008 Apr 1;44(7):1323-1333.

(**2**) Lee S, Lee SJ, Coronata AA, Fredenburgh L, Chung SW, Perrella M, et al. Carbon Monoxide Confers Protection in Sepsis by Enhancing Beclin 1-Dependent Autophagy and Phagocytosis. Antioxid Redox Signal 2013 Aug 25.

(**3**) Onyiah JC, Sheikh SZ, Maharshak N, Steinbach EC, Russo SM, Kobayashi T, et al. Carbon monoxide and heme oxygenase-1 prevent intestinal inflammation in mice by promoting bacterial clearance. Gastroenterology 2013 Apr;144(4):789-798.

(**4**) Chiang N, Shinohara M, Dalli J, Mirakaj V, Kibi M, Choi AM, et al. Inhaled carbon monoxide accelerates resolution of inflammation via unique proresolving mediator-heme oxygenase-1 circuits. J Immunol 2013 Jun 15;190(12):6378-6388.

(**5**) Tacke F. Functional role of intrahepatic monocyte subsets for the progression of liver inflammation and liver fibrosis in vivo. Fibrogenesis Tissue Repair 2012 Jun 6;5 Suppl 1:S27.

(**6**) Pradere JP, Kluwe J, De Minicis S, Jiao JJ, Gwak GY, Dapito DH, et al. Hepatic macrophages but not dendritic cells contribute to liver fibrosis by promoting the survival of activated hepatic stellate cells in mice. Hepatology 2013 Oct;58(4):1461-1473.

(**7**) Dudnik LB, Khrapova NG. Characterization of bilirubin inhibitory properties in free radical oxidation reactions. Membr Cell Biol 1998;12(2):233-240.

(**8**) Jaeschke H, Ho YS, Fisher MA, Lawson JA, Farhood A. Glutathione peroxidase-deficient mice are more susceptible to neutrophil-mediated hepatic parenchymal cell injury during endotoxemia: importance of an intracellular oxidant stress. Hepatology 1999 Feb;29(2):443-450.

(**9**) Lei XG. Glutathione peroxidase-1 gene knockout on body antioxidant defense in mice. Biofactors 2001;14(1-4):93-99.

(**10**) Yoshida T, Watanabe M, Engelman DT, Engelman RM, Schley JA, Maulik N, et al. Transgenic mice overexpressing glutathione peroxidase are resistant to myocardial ischemia reperfusion injury. J Mol Cell Cardiol 1996 Aug;28(8):1759-1767.

(**11**) Han D, Hanawa N, Saberi B, Kaplowitz N. Mechanisms of liver injury. III. Role of glutathione redox status in liver injury. Am J Physiol Gastrointest Liver Physiol 2006 Jul;291(1):G1-7.

(**12**) Cesaratto L, Vascotto C, Calligaris S, Tell G. The importance of redox state in liver damage. Ann Hepatol 2004 Jul-Sep;3(3):86-92.

(**13**) Garcia-Ruiz C, Fernandez-Checa JC. Redox regulation of hepatocyte apoptosis. J Gastroenterol Hepatol 2007 Jun;22 Suppl 1:S38-42.

(**14**) Zhong Z, Froh M, Wheeler MD, Smutney O, Lehmann TG, Thurman RG. Viral gene delivery of superoxide dismutase attenuates experimental cholestasis-induced liver fibrosis in the rat. Gene Ther 2002 Feb;9(3):183-191.

(**15**) Wheeler MD, Nakagami M, Bradford BU, Uesugi T, Mason RP, Connor HD, et al. Overexpression of manganese superoxide dismutase prevents alcohol-induced liver injury in the rat. J Biol Chem 2001 Sep 28;276(39):36664-36672.

(**16**) Chen Y, Choi SS, Michelotti GA, Chan IS, Swiderska-Syn M, Karaca GF, et al. Hedgehog controls hepatic stellate cell fate by regulating metabolism. Gastroenterology 2012 Nov;143(5):1319-29.e1-11.

(**17**) Oberley LW, Buettner GR. Role of superoxide dismutase in cancer: a review. Cancer Res 1979 Apr;39(4):1141-1149.

(**18**) Klimova T, Chandel NS. Mitochondrial complex III regulates hypoxic activation of HIF. Cell Death Differ 2008 Apr;15(4):660-666.

(**19**) Kim JW, Gao P, Liu YC, Semenza GL, Dang CV. Hypoxia-inducible factor 1 and dysregulated c-Myc cooperatively induce vascular endothelial growth factor and metabolic switches hexokinase 2 and pyruvate dehydrogenase kinase 1. Mol Cell Biol 2007 Nov;27(21):7381-7393.

(**20**) Semenza GL. Oxygen-dependent regulation of mitochondrial respiration by hypoxia-inducible factor 1. Biochem J 2007 Jul 1;405(1):1-9.

(**21**) Semenza GL. Hypoxia and cancer. Cancer Metastasis Rev 2007 Jun;26(2):223-224.
(**22**) Wang Y, Huang Y, Guan F, Xiao Y, Deng J, Chen H, et al. Hypoxia-Inducible Factor-1alpha and MAPK Co-Regulate Activation of Hepatic Stellate Cells upon Hypoxia Stimulation. PLoS One 2013 Sep 10;8(9):e74051.
(**23**) Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 2009 May 22;324(5930):1029-1033.
(**24**) Gough DR, Cotter TG. Hydrogen peroxide: a Jekyll and Hyde signalling molecule. Cell Death Dis 2011 Oct 6;2:e213.
(**25**) Dunning S, Hannivoort RA, de Boer JF, Buist-Homan M, Faber KN, Moshage H. Superoxide anions and hydrogen peroxide inhibit proliferation of activated rat stellate cells and induce different modes of cell death. Liver Int 2009 Jul;29(6):922-932.
(**26**) Sarsour EH, Venkataraman S, Kalen AL, Oberley LW, Goswami PC. Manganese superoxide dismutase activity regulates transitions between quiescent and proliferative growth. Aging Cell 2008 Jun;7(3):405-417.
(**27**) Molloy JW, Calcagno CJ, Williams CD, Jones FJ, Torres DM, Harrison SA. Association of coffee and caffeine consumption with fatty liver disease, nonalcoholic steatohepatitis, and degree of hepatic fibrosis. Hepatology 2012 Feb;55(2):429-436.
(**28**) Freedman ND, Everhart JE, Lindsay KL, Ghany MG, Curto TM, Shiffman ML, et al. Coffee intake is associated with lower rates of liver disease progression in chronic hepatitis C. Hepatology 2009 Nov;50(5):1360-1369.
(**29**) Modi AA, Feld JJ, Park Y, Kleiner DE, Everhart JE, Liang TJ, et al. Increased caffeine consumption is associated with reduced hepatic fibrosis. Hepatology 2010 Jan;51(1):201-209.
(**30**) Birerdinc A, Stepanova M, Pawloski L, Younossi ZM. Caffeine is protective in patients with non-alcoholic fatty liver disease. Aliment Pharmacol Ther 2012 Jan;35(1):76-82.
(**31**) Moreno MG, Chavez E, Aldaba-Muruato LR, Segovia J, Vergara P, Tsutsumi V, et al. Coffee prevents CCl(4)-induced liver cirrhosis in the rat. Hepatol Int 2011 Sep;5(3):857-863.
(**32**) Furtado KS, Prado MG, Aguiar E Silva MA, Dias MC, Rivelli DP, Rodrigues MA, et al. Coffee and caffeine protect against liver injury induced by thioacetamide in male Wistar rats. Basic Clin Pharmacol Toxicol 2012 Nov;111(5):339-347.
(**33**) Klemmer I, Yagi S, Gressner OA. Oral application of 1,7-dimethylxanthine (paraxanthine) attenuates the formation of experimental cholestatic liver fibrosis. Hepatol Res 2011 Nov;41(11):1094-1109.
(**34**) Lv X, Chen Z, Li J, Zhang L, Liu H, Huang C, et al. Caffeine protects against alcoholic liver injury by attenuating inflammatory response and oxidative stress. Inflamm Res 2010 Aug;59(8):635-645.
(**35**) Gressner OA, Lahme B, Siluschek M, Gressner AM. Identification of paraxanthine as the most potent caffeine-derived inhibitor of connective tissue growth factor expression in liver parenchymal cells. Liver Int 2009 Jul;29(6):886-897.
(**36**) Fredholm BB, Battig K, Holmen J, Nehlig A, Zvartau EE. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacol Rev 1999 Mar;51(1):83-133.
(**37**) Chan ES, Liu H, Fernandez P, Luna A, Perez-Aso M, Bujor AM, et al. Adenosine A2A receptors promote collagen production by a Fli1- and CTGF-mediated mechanism. Arthritis Res Ther 2013 May 11;15(3):R58.
(**38**) Chan ES, Montesinos MC, Fernandez P, Desai A, Delano DL, Yee H, et al. Adenosine A(2A) receptors play a role in the pathogenesis of hepatic cirrhosis. Br J Pharmacol 2006 Aug;148(8):1144-1155.
(**39**) Che J, Chan ES, Cronstein BN. Adenosine A2A receptor occupancy stimulates collagen expression by hepatic stellate cells via pathways involving protein kinase A, Src, and extracellular signal-regulated kinases 1/2 signaling cascade or p38 mitogen-activated protein kinase signaling pathway. Mol Pharmacol 2007 Dec;72(6):1626-1636.
(**40**) Deree J, Martins JO, Melbostad H, Loomis WH, Coimbra R. Insights into the regulation of TNF-alpha production in human mononuclear cells: the effects of non-specific phosphodiesterase inhibition. Clinics (Sao Paulo) 2008 Jun;63(3):321-328.
(**41**) Rieckmann P, Weber F, Gunther A, Martin S, Bitsch A, Broocks A, et al. Pentoxifylline, a phosphodiesterase inhibitor, induces immune deviation in patients with multiple sclerosis. J Neuroimmunol 1996 Feb;64(2):193-200.
(**42**) Zhang D, Jiang H, Wang Y, Ma J. Pentoxifylline inhibits hepatic stellate cells proliferation via the Raf/ERK pathway. APMIS 2012 Jul;120(7):572-581.

(**43**) Hernandez E, Bucio L, Souza V, Escobar MC, Gomez-Quiroz LE, Farfan B, et al. Pentoxifylline downregulates alpha (I) collagen expression by the inhibition of Ikappabalpha degradation in liver stellate cells. Cell Biol Toxicol 2008 Aug;24(4):303-314.

(**44**) Romanelli RG, Caligiuri A, Carloni V, DeFranco R, Montalto P, Ceni E, et al. Effect of pentoxifylline on the degradation of procollagen type I produced by human hepatic stellate cells in response to transforming growth factor-beta 1. Br J Pharmacol 1997 Nov;122(6):1047-1054.

(**45**) Windmeier C, Gressner AM. Pharmacological aspects of pentoxifylline with emphasis on its inhibitory actions on hepatic fibrogenesis. Gen Pharmacol 1997 Aug;29(2):181-196.

(**46**) Tyagi P, Sharma P, Sharma BC, Puri AS, Kumar A, Sarin SK. Prevention of hepatorenal syndrome in patients with cirrhosis and ascites: a pilot randomized control trial between pentoxifylline and placebo. Eur J Gastroenterol Hepatol 2011 Mar;23(3):210-217.

(**47**) Cavin C, Holzhaeuser D, Scharf G, Constable A, Huber WW, Schilter B. Cafestol and kahweol, two coffee specific diterpenes with anticarcinogenic activity. Food Chem Toxicol 2002 Aug;40(8):1155-1163.

(**48**) Upadhyay R, Mohan Rao LJ. An outlook on chlorogenic acids-occurrence, chemistry, technology, and biological activities. Crit Rev Food Sci Nutr 2013;53(9):968-984.

(**49**) Shan J, Fu J, Zhao Z, Kong X, Huang H, Luo L, et al. Chlorogenic acid inhibits lipopolysaccharide-induced cyclooxygenase-2 expression in RAW264.7 cells through suppressing NF-kappaB and JNK/AP-1 activation. Int Immunopharmacol 2009 Aug;9(9):1042-1048.

Nederlandse samenvatting

Chronische leverziekten zijn een grote last voor de gezondheidszorg; ze komen vrij vaak voor en gaan gepaard met ernstige morbiditeit die veel ziekenhuisopnames vereist. Er zijn verschillende oorzaken voor chronische leverziekten. De meest bekende zijn virale hepatitis en alcohol-geïnduceerde leverschade, maar ook obesitas leidt tot leverziekte door leververvetting (steatose). Ongeacht de oorzaak van de leverziekte, leidt het vaak tot verbindweefseling van de lever, fibrose, en het laatste stadium van deze leverziekte is levercirrose.

De verbindweefseling van de lever ontstaat uit een wondgenezingsreactie. Door de schadelijke stimulus gaan de functionele levercellen (de hepatocyten) dood en er treedt een ontstekingsreactie op om de schade te beperken. Daarbij worden myofibroblasten (stellaatcellen, portale myofibroblasten) geactiveerd die littekenweefsel produceren. In de acute, kortdurende fase is dit een gunstige reactie, die ervoor zorgt dat een wondje weer dichtgaat en geneest. Echter, als er aanhoudende schade is, kan er een chronische wondgenezingsreactie ontstaan. Deze voortdurende ontstekingsreactie zorgt voor een aanhoudende stimulatie van myofibroblasten, terwijl er steeds meer hepatocyten doodgaan. Dit wordt de lever-paradox genoemd: in dezelfde omgeving gaan hepatocyten dood, maar de myofibroblasten delen en produceren volop littekenweefsel.

Een belangrijke component van de chronische ontstekingsreactie is oxidatieve stress. Oxidatieve stress wordt gedefinieerd als een teveel aan zuurstofradicalen, en daardoor een verstoring van het normale evenwicht tussen pro- en antioxidanten. Zuurstofradicalen zijn onder andere waterstofperoxide en superoxide anionen. Deze kunnen ontstaan in fysiologische omstandigheden (bijvoorbeeld bij het normale celmetabolisme en het gebruik van zuurstof), maar ook in pathologische omstandigheden. In pathologische omstandigheden worden zuurstofradicalen geproduceerd door ontstekingscellen zoals macrofagen (Kupffer cellen), maar ook bij het doodgaan van hepatocyten of bij het metabolisme van alcohol of paracetamol komen zuurstofradicalen vrij.

Een cel is normaal uitgerust met een aantal antioxidantmechanismen. Deze zijn in staat om zuurstofradicalen te neutraliseren. Allereerst is er de familie van superoxide dimsutases (SODs), deze zetten superoxide anionen om in waterstofperoxide. Er zijn een aantal vormen van, koper-zink SOD (of CuZnSOD) bevindt zich in het cytoplasma van de cel en mangaan SOD (MnSOD) is gelokaliseerd in de mitochondriën van de cel. Waterstofperoxide wordt vervolgens weer omgezet in water en zuurstof door andere enzymen, namelijk catalase en glutathione peroxidase.

Om de lever paradox te kunnen verklaren is onze veronderstelling dat er een aanpassingsvermogen is van de myofibroblasten (voornamelijk afkomstig van de stellaatcellen). Mogelijk dat dit een aanpassing betreft van de antioxidantstatus van de cellen, waardoor ze beter bestand zijn tegen schadelijke invloeden.

Het doel van dit proefschrift was om de mechanismen van stellaatcel activatie te onderzoeken. Hierbij hebben we ons met name gericht op de rol van oxidatieve stress en anti-oxidant mechanismen in deze cellen. Ons uiteindelijke doel was een bijdrage leveren aan een therapie specifiek gericht op de stellaatcel als veroorzaker van lever fibrose.

In **hoofdstuk 2** hebben we de rol van heme oxygenase-1 (HO-1) onderzocht in het ontstaan van fibrose. We hebben in een *in vivo* diermodel van cholestatische leverziekte, het galgangligatie (BDL: bile duct ligation) model, gekeken naar de expressie van HO-1. HO-1 kwam enkel tot expressie in macrofagen (ED-1 positieve cellen).

HO-1 is vervolgens kunstmatig tot overexpressie gebracht in stellaatcellen en in macrofagen. In macrofagen met een verhoogd niveau van HO-1 expressie waren de merkers verhoogd die geassocieerd zijn met het pro-inflammatoire M1 fenotype van macrofagen. De merkers geassocieerd met het M2 fenotype, geassocieerd met littekenvorming en weefsel remodellering, waren in expressieniveau gedaald na HO-1 inductie. In stellaatcellen zorgde HO-1 inductie niet voor een verandering van het activatieniveau.

Hieruit kunnen we concluderen dat HO-1 in staat is om het fenotype van de macrofagen te veranderen. Deze kunnen op hun beurt mogelijk weer andere cellen beïnvloeden, bijvoorbeeld myofibroblasten en stellaatcellen. Hier moet nog verder onderzoek naar gedaan worden. Het feit dat HO-1 zorgt voor een pro-inflammatoir fenotype van de macrofagen lijkt op het eerste gezicht ongunstig, omdat ontsteking vaak wordt gekoppeld aan meer schade en daardoor meer fibrose. Echter, wij veronderstellen dat een initiële inductie van ontsteking gunstig is, omdat het zorgt voor een snelle eliminatie van de schadelijke stimulus. Dit zal op de langere termijn minder activatie geven van de stellaatcellen en dus minder fibrose. Bovendien zal een vermindering van het aantal macrofagen met het M2 fenotype zorgen voor een minder pro-fibrotische omgeving.

In **hoofdstuk 3** hebben we de beschermende mechanismen van geactiveerde stellaatcellen tegen oxidatieve stress-geïnduceerde celdood onderzocht. Als eerste

hebben we de anti-oxidantprofielen van stellaatcellen bekeken, en rustende stellaatcellen vergeleken met geactiveerde stellaatcellen. Hieruit bleek dat er een aantal verschillen waren tussen rustende en geactiveerde stellaatcellen. De niveaus van glutathione en glutathione peroxidase (GPx1) stegen wanneer de stellaatcellen actief werden, terwijl de expressieniveaus van catalase en MnSOD dramatisch daalden.

Daarna hebben we geactiveerde stellaatcellen blootgesteld aan waterstofperoxide en gekeken naar mechanismen van celdood: apoptose, de gereguleerde vorm van celdood, of necrose, de niet-gereguleerde vorm. Geactiveerde stellaatcellen zijn resistent tegen waterstofperoxide-geïnduceerde celdood. Echter, indien de intracellulaire glutathione concentratie wordt verlaagd, zorgt waterstofperoxide voor necrose van de stellaatcellen. Remming van de activiteit van GPx1 en catalase (tegelijk en separaat) zorgt echter voor waterstofperoxide-geïnduceerde apoptose.

De inductie van glutathione en GPx1 zijn een duidelijk aanpassingsmechanisme van de actieve stellaatcellen, en vooral glutathione lijkt onmisbaar te zijn. GPx1 en catalase zijn beiden verantwoordelijk voor het neutraliseren van waterstofperoxide, en waar GPx1 geïnduceerd wordt bij stellaatcelactivatie, gaat catalase omlaag. Het remmen van beiden enzymen leidt tot apoptose van de stellaatcellen na blootstelling aan waterstofperoxide. Blijkbaar zijn ze toch even belangrijk bij het neutraliseren van waterstofperoxide. Voor apoptose lijkt glutathione noodzakelijk, en waarschijnlijk komt dit door de bepalende rol die glutathione speelt in het handhaven van de redox status van de cel.

De studie in hoofdstuk 3 toont een aanpassingsmechanisme van stellaatcellen die zij verwerven tijdens hun activatie. De verhoging van intracellulaire glutathione spiegels maakt de cellen meer resistent tegen necrose, hoewel ze het vermogen om in apoptose te gaan behouden. Dit kan een verklaring zijn voor het verschijnsel dat stellaatcellen in apoptose gaan tijdens resolutie van fibrose.

De afname van MnSOD expressie in geactiveerde stellaatcellen lijkt niet te passen in de hypothese dat de stellaatcellen zich aanpassen aan een vijandige pro-oxidant omgeving. Zuurstofradicalen die geproduceerd worden tijdens leverfibrose zijn o.a. waterstofperoxide, superoxide anionen en hydroxylradicalen. Blijkbaar zijn stellaatcellen aangepast om beter om te kunnen gaan met waterstofperoxide, hoewel hun verdediging tegen superoxide anionen zwak lijkt met een verlaagde expressie van MnSOD en een stabiel blijvende expressie van CuZnSOD.

In **hoofdstuk 4** hebben we de rol van MnSOD in stellaatcellen verder onderzocht, en in het bijzonder hebben we gekeken naar de mogelijkheid van het terugdraaien van de activatie van stellaatcellen door de expressie van MnSOD te verhogen.

MnSOD is tot overexpressie gebracht met behulp van een adenovirale vector in stellaatcellen. Deze overexpressie leidde tot een verlaging van merkers die geassocieerd zijn met geactiveerde stellaatcellen, en een verhoging van de merker die geassocieerd is met rustende stellaatcellen. Naast de veranderingen in deze merkers, waren de stellaatcellen ook fenotypisch veranderd. Ze waren kleiner en het typische filament netwerk was veel minder uitgesproken in de cellen die meer MnSOD hadden.

Een inductie van MnSOD zou een betere bescherming betekenen van de mitochondrieën tegen superoxiden. Dit lijkt in tegenspraak met de hypothese dat geactiveerde stellaatcellen beter beschermd zijn tegen oxidatieve stress, omdat deze cellen juist weinig MnSOD hebben. Er zijn een aantal mogelijke verklaringen voor deze tegenstelling.

Ten eerste is bekend dat stellaatcellen tijdens hun activatieproces omschakelen naar een ander metabolisme. Als rustende cellen hebben ze een 'normaal' metabolisme van oxidatieve fosforylering als onderdeel van de citroenzuurcyclus, waar zuurstof gebruikt wordt om uiteindelijk ATP (een zogeheten energiedrager) te produceren. Echter, als de stellaatcellen actief worden, verandert het metabolisme in aërobe glycolyse, waarbij glucose wordt afgebroken zonder dat daarna de citroenzuurcyclus volgt. Dit wordt ook gezien in kankercellen en dit wordt het Warburg effect genoemd. Een ander kenmerk van dit Warburg effect is dat het aantal mitochondriën niet zoals verwacht afneemt, omdat er geen oxidatieve fosforylering meer is, maar juist toeneemt. Het is bekend dat kankercellen op dit metabolisme overschakelen, omdat het weinig zuurstof vereist. Mogelijk is dit ook het geval in stellaatcellen die activatie ondergaan. De hoge metabole activiteit die hierbij vereist is kan mogelijk niet opgebracht worden door oxidatieve fosforylering. Een ander punt is dat glycolyse ook besparend is ten opzichte van oxidatieve fosforylering, want er zijn veel grondstoffen nodig om de 36 ATP moleculen per glucosemolecuul te maken in de oxidatieve fosforylering in plaats van de 4 ATP moleculen per glucosemolecuul in glycolyse. De overgebleven biomassa in de glycolyse kan gebruikt worden voor de synthese van andere materialen, bijvoorbeeld de productie van extracellulaire matrix. Als MnSOD wordt geïnduceerd, zoals in onze studie is gebeurd, zien we dat de cellen meer kenmerken van rustende stellaatcellen krijgen, mogelijk gaat dit gepaard of wordt dit zelfs gestuurd door een verandering van metabolisme.

Een ander interessant concept, dat de verlaging van MnSOD kan verklaren, is de mogelijk redoxcontrole van de celcyclus. Eerdere studies in embryonale fibroblasten laten zien, dat een verlaging van MnSOD leidt tot een verhoging van het aantal superoxide anionen en minder waterstofperoxide, en dit geassocieerd is met deling van de cellen. Andersom was een verhoging van MnSOD geassocieerd met het verlaten van de celcyclus. Of dit ook in onze studieopzet werkt, zal verder onderzocht moeten worden.

Tot slot hebben we in **hoofdstuk 5** een toepasbare manier van fibrogenese-remming onderzocht. Geactiveerde stellaatcellen en Kupffer cellen zijn geïncubeerd met caffeine en een metaboliet van caffeine: 1,7-dimethylxanthine (1,7-DMX). Met deze behandeling bleken stellaatcellen minder actief te worden: zowel de merkers van actieve stellaatcellen als de proliferatie verminderde. Het effect van caffeine en 1,7-DMX bleek onafhankelijk van elkaar aanwezig, maar werd versterkt als beide stoffen tegelijk aanwezig waren. Dit wijst erop dat er zowel directe als indirecte effecten zijn van caffeine.

Caffeine wordt normaal gesproken gemetaboliseerd door een enzym in de hepatocyten (cytochrome P450 $2^{E}1$: CYP2E1). Dit enzym is niet aanwezig in stellaatcellen en ook nauwelijks in Kupffer cellen. Aangezien er wel effecten zijn van caffeine op stellaatcellen, is er waarschijnlijk een direct effect van caffeine. Uit de literatuur is bekend dat caffeine ook kan werken als adenosine receptor antagonist (in lage dosis) en in hogere dosis kan werken als een fosfodiësterase-remmer (wat remming geeft van de ontstekingsreactie). Echter 1,7-DMX heeft ook een antifibrotisch effect op stellaatcellen, wat aangeeft dat caffeine ook een indirect effect heeft op stellaatcellen. 1,7-DMX is één van de metabolieten van caffeine, andere metabolieten theobromine en theofylline hebben ook een remmende werking op het fosfodiësterase systeem met als effect remming van de ontsteking.

Kupffer cellen (de macrofagen uit de lever) behandeld met caffeine en 1,7-DMX lieten een verminderde LPS-geïnduceerde productie van het inflammatoire cytokine (TNF-α) zien. Ook lieten hepatocyten die behandeld werden met 1,7-DMX een verlaagde iNOS productie zien (een maat voor ontsteking) na stimulatie met een pro-inflammatoire cytokine mix (IL-1β, interferon-γ en TNF-α). Dit geeft aan dat caffeine in de gehele lever kan zorgen voor een vermindering van de ontsteking.

Concluderend heeft caffeine zowel directe als indirecte antifibrotische effecten, via remming van de stellaatcelactivatie en via remming van de ontsteking. De werking van caffeine is duaal: direct via remming van de ontsteking en indirect via 1,7-DMX.

Hoofdstuk 6 beschrijft tot slot een samenvatting van het proefschrift met een bespiegeling over eventuele klinische toepassingen in de toekomst. In dit proefschrift is enkel onderzoek verricht met cellen, waardoor de klinische toepassing wellicht niet direct duidelijk is. Dit proefschrift laat zien dat er verschillende therapieën zijn die zowel gunstig zijn voor de hepatocyten als voor andere cellen van de lever (bijvoorbeeld de stellaatcellen en de Kupffer cellen), zoals HO-1 inductie, MnSOD inductie of een behandeling met caffeine. Deze therapieën, die verschillende effecten hebben, zijn het waard om verder uitgewerkt te worden zodat ze uiteindelijk een klinische toepassing kunnen hebben.

Dankwoord

Eindelijk is het zover… HET IS AF!

Het leek bij vlagen nooit af te komen, weer een mislukt experiment, weer een isolatie met weinig cellen... Maar uiteindelijk met veel hulp is het dan toch gelukt.

Als eerste ben ik daarvoor veel dank verschuldigd aan mijn promotor: Prof. Dr. A.J. Moshage. Beste Han, ongelooflijk hoe jij altijd er weer een positieve draai aan kan geven, als je niet fysiek aanwezig was dan wel via de mail. Als ik door de bomen het bos niet meer zag, keerde ik na een gesprek met jou weer vol moed en enthousiasme terug naar het lab.

Dan mijn tweede promotor, Prof. Dr. K.N. Faber, beste Klaas Nico, hoe vaak heb ik bij jou om de deur gekeken of Han er was... Voor een groot gedeelte van mijn promotietraject was jij het eerste aanspreekpunt voor mij, altijd in om even door de microscoop te kijken naar rare kleuringen. Heel erg veel dank voor de discussies tijdens de labmeetings.

Bij dezen wil ik ook de leden van mijn leescommissie, Prof. Dr. H. Van Goor en Prof. Dr. Henning, erg bedanken voor het lezen van mijn proefschrift. También muchas gracias a Prof. Dr. M. Arrese por leer mi tesis!

Hoe kon ik als geneeskundestudent, die stiekum veel meer alpha is dan bèta, zo'n proefschrift bij elkaar pipetteren? Onmogelijk zonder de hulp van de analisten van de MDL...

Allereest Manon, van leren pipetteren tot hulp bij de laatste experimenten, van hulp bij berekeningen tot hulp bij de isolaties.. Heel erg bedankt! Super dat je ook me bij het laatste onderdeel van dit traject wil bijstaan als paranimf! Tjasso, heel erg bedankt voor alle vroege koffiemomentjes, hulp bij de confocale microscoop en advies over vanalles en nog wat. Het is altijd gezellig om even een praatje met je te maken, vooral op vrijdagmiddag als er niemand anders lijkt te werken! Janette, heel erg bedankt voor je adviezen bij kleuringen en qPCR. Bojana, bedankt voor de leuke tijd die we samen hebben gehad, samen strijdend op het CDP tegen allerlei mislukte isolaties... Heel veel succes de komende tijd! Fiona, Mariska en Haukeline, ook jullie erg bedankt voor jullie hulp.

Dan mijn collega-PhD studenten van de MDL, wat een tijd hebben we beleefd.. Esther, samen begonnen als studenten en nu zijn we allebei PhD studenten in het Moshage-Faber lab. Met jou is het altijd lachen, van samen in het lab Floris pesten met te kleine handschoenen tot congresbezoeken in San Fransisco en niet te vergeten Veldhoven en Spier. Ook zal ik nooit 'the morning after' het Land van Belofte vergeten… Gelukkig hebben we de foto's nog! Dank dat je voor mij een jurk aantrekt als paranimf zijnde! Mark, wat een moeilijke tijd heb je achter de rug, tijd om vooruit te kijken en verder te gaan! Heel veel succes hiermee! Golnar, ik vond het heel erg fijn dat jij mijn kamergenoot was in het begin van mijn PhD! Omdat Han er vaak niet was, moesten we ons met z'n tweeën redden, en jij hebt me heel veel geleerd! Succes met je Nederlandse doktercarrière! Floris, als collega MD / PhD bij de MDL hebben we elkaar niet veel gezien, telkens afgewisseld in de kliniek en op het lab. Dank voor je niet aflatende enthousiasme en veel succes met het afronden van alles! Jackey, it was really nice to have you in the lab for two years. Thank you for explaining some experiments to me and thanks for our nice discussions and talks. Goodluck finishing your thesis in China! Atta, although we had our disagreements (for example about throwing away or passing cells ..:)) you always made me smile with your singing and your creative ideas. Good luck with your career! Anouk, dank je wel voor je vrolijkheid op het lab! Veel succes met het afronden van je proefschrift en veel geluk met je gezinnetje! Shiva, thank you for our nice talks! Goodluck with your research and enjoy your cat! ☺ Ali and Danial, new members of the lab, I wish you all the best with your projects and Dutch lives!

Oudleden van het MDL lab: Sandra, Martijn, Axel, Titia, Jannes, Rohina en Elise, dank voor de tijd dat we hebben samengewerkt!

Natuurlijk dank aan mijn studenten Marja Haakma-de Haan en Fernanada Braga Seganfredo voor hun inzet en gezelligheid! Thank you!

Dank aan mijn kamergenoten boven het lab, vaak een oase van rust: Nienke (succes met het afronden van je proefschrift en met je nieuwe baan!), Jan Freark (succes met je leven na de verdediging!), Weilin (dank je wel voor de nederlandse gesprekken!), Mathijs (succes met je project!), Maaike, Mariëtte, Maxi.

Iedereen die werkt(e) bij kindergeneeskunde (aka LDS): Andrea, Aycha, Angelika, Bert, Renze, Henk, Niels, Danny, Dicky, Henk Jan, Gijs, Lydia, Hans, Marcella, Marije, Arne, Brenda, Vera, Nicoline, Karen, Carolien, Marleen, Gemma, Wytske, Margot, Maurien, Otto, Gerard, Trijnie, Theo, Uwe, Vincent, Tim, Janine, Dolf, Hilde en Gea.

Dank aan de werkgroep Junior Scientific Masterclass voor het honoreren van mijn MD/PhD project en de voorafgaande cursussen, in het bijzonder Michiel Hooiveld, Hanneke Kluin-Nelemans, Joke van der Mark en Robert Porte.

Veel dank aan mijn lieve vrienden en vriendinnen, waar ik niet veel tijd voor heb gehad, maar waar ik toch altijd lekker mee kon ontspannen en leuke dingen doen. Dank je wel Elise (voor het samen sporten) en Gerard, Myrthe (voor weekendjes Delft en met zn drieën naar de sauna opwarmen), Joke (voor gezellig kletsen en de support), Charlotte en Lucas (voor de gezellige avonden weddingplannen, kan niet wachten...), Jan, Jasper en Esther (mijn mede MD/PhDers en borrelaars uit Leeuwarden, altijd veel plezier gehad met jullie op borrels waar dan ook georganiseerd!), Josephine (mijn buddy-MD/PhD, tegelijk begonnen en tegelijk klaar! We did it!), Jop en Bas (dank voor alle KEI-2005 reünieën), Judith en Kyrill (succes met het afronden van jullie boekjes!).

Dank aan mijn lieve familie, altijd was het onduidelijk waar ik me nu weer mee bezig hield, en was ik nou nog steeds niet afgestudeerd..? Eindelijk is het klaar, het onderzoek is af en volgende maand ben ik ook afgestudeerd..

Dank je wel lieve Opa en Oma Vincent, lieve Ans, Erik & Judith en Mathijs. Een betere bijna-schoonfamilie kan ik mij niet wensen.

Lieve Oma, Sisy en Ed, Boy, Hans en Daan, Roos en Fee, Judith en Arjen (+Noa) dank je wel dat jullie zo'n lieve familie voor me zijn! Vooral het kerstdiner in 2012 vond ik heel erg geslaagd!

Lieve Ingrid en Tanno, dank jullie wel voor het vertrouwen in mij!

Lieve Maurits, Hilde, Anne, Joris, Jelle en Charlotte, wat is het leuk om jullie grote zus te zijn, altijd gezelligheid met spelletjesavonden. Ik zou heel graag willen dat we dat vasthouden nu ieder zijn eigen leventje opbouwt!

Lieve Papa en Nynke, heel erg bedankt voor al jullie steun. Ik geniet van alle discussies, inhoudelijk over het werk, maar ook over algemene dingen. Bij jullie kan ik altijd wat kwijt, en jullie helpen me vaak weer de goede kant uit. Nu dit allemaal klaar is hebben jullie een etentje verdiend!

Thomas, mijn allerliefste schat, beste vriend, steun en toeverlaat. Heel erg bedankt voor alle opbeurende gesprekken als ik er weer eens helemaal doorheen zat... Voordat ik hier aan begon, 5 jaar geleden, zei ik al dat het niet gemakkelijk zou worden, maar zoals alles hebben we dit ook weer overleefd. Ik kan niet wachten tot ik officieel Mw. Tilkema-Tiebosch word!

Curriculum Vitae

Marjolein Tiebosch werd op 29 juli 1987 geboren in Maastricht. Na het voltooien van haar gymnasium examen aan het Maartenscollege te Haren in 2005, werd zij middels loting toegelaten tot de opleiding Geneeskunde aan de Rijksuniversiteit Groningen.

Tijdens haar bachelor kwam ze reeds in aanraking met het onderzoek op het maag-, darm-, en leverziektenlaboratorium van Prof. Dr. A.J. Moshage en Prof. Dr. Faber.

Na op dit lab haar scriptie voor de studie Geneeskunde te hebben voltooid, begon ze aan haar eerste jaar co-schappen in het UMCG. Tijdens dit jaar werd zij aangenomen voor een MD/PhD-traject op het lab MDL, welke startte in mei 2010. Van mei 2011 tot mei 2012 deed zij haar tweede jaar co-schappen in het Medisch Centrum te Leeuwarden. Hierna keerde zij terug naar het lab om haar MD/PhD traject af te ronden. Na het inleveren van het proefschrift aan de leescommissie in oktober 2013 begon zij aan haar semi-artsstage op de afdeling Longziekten en Tuberculose in het UMCG, met als verdiepingsstage Longoncologie. Dit rondt zij eind maart 2014 af, waarna ze dit proefschrift zal verdedigen in april 2014.